MATING BIOLOGY OF *AEDES AEGYPTI* MALES

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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January 2013

ABSTRACT

The objectives of this study were to develop first an accessory gland specific promoter and to investigate some basic aspects of the mating biology of *Aedes aegypti,* an important vector of dengue and dengue hemorrhagic fever. In addition, I investigated male ejaculate allocation patterns as influenced by female reproductive quality (female body size), and I studied male mating preferences in semi-field conditions in a dengue endemic region of Thailand.

I developed an AG-specific promoter construct for male *Ae. aegypti* that can be used in future studies targeting seminal fluid protein (sfp) genes and understanding sfp function. I first identified the 5´ UTR of AAEL010824 gene with a 5´ UTR average length of 22 bases. I then cloned the region 5 kb upstream this gene and incorporated it into a DNA plasmid pBac[3xP3-DsRedaf] construct to obtain the first AG-specific promoter pBac[3xP3-AAEL010824EGFP-DsRedaf] for *Ae. aegypti*.

In this study, I explored whether *Ae. aegypti* males modulate the quantity of sperm and seminal fluid proteins (AAEL010824). Evidence suggested that *Ae. aegypti* males transfer greater sperm to large females than small ones. I found no evidence of significant differences in sperm or sfp allocation by males under different mating competition conditions. In addition, there was no significant difference in the amount of sfp determined by quantitation of AAEL010824 transferred to females regardless of size or competition from other males.

In the third component of my research project, I investigated male mating preferences for females by body size. Body size is known to be directly related to

fecundity in female mosquitoes and is an important component of fitness. Although my samples sizes were low, I found some indication that mate choice by size may occur in *Ae. aegypti*. However, more experiments need to be conducted to definitively address this question.

Collectively, this work was intended to contribute to a greater understanding of mosquito mating biology as it relates to the use of transgenic males for population control programs.

BIOGRAPHICAL SKETCH

Prasit Deewatthanawong was born on June 2nd 1972 in Bangkok, the capital city of Thailand. He graduated a Agriculture Bachelors degree from King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand. He earned a bachelor's degree with pest management technology major and his senior project focused on "Studies on some medicinal plants for controlling Cowpea Weevil (*Callosobruchus maculates* F.)". After graduation in 1994, he continued to study a M.S. in biotechnology at Kasetsart University, Thailand. He earned his M.S. degree in 1998 with his distinction thesis in the topic "Studies on growth, sporulation, and enzyme activity of *Bacillus thuringiensis*". He started first working career as a sale representative for scientific equipment and he had opportunity changing my career as a project management officer at The Thailand Research Fund (TRF) where he was in charge of reviewing and evaluating funded agricultural projects. He later had an opportunity to work independently on his own research after he decided to take another job as a lecturer in the Department of Applied Biology at KMITL. However, he relocated to the United States with his wife, who was accepted as a doctoral student at Cornell University in 2003. Since coming to the United States, he had been part of a volunteer in a plant science lab at Cornell and had a great chance that Boyce Thompson Institute for Plant Research (BTI) offered him a research technician position which allows him to work closely and intensively with plants. At BIT, he began to work on mechanisms of development of the symbiosis and symbiotic phosphate transport system in *Medicago truncatula* at the Harrison lab and relocated to work on a project entitled "A two component A*ctivator/ Dissociation* platform for reverse and forward genetic analysis in maize" at the Brutnell lab. He joined the Harrington lab, the Department of

Entomology, Cornell University as a research assistant and interested in applying molecular techniques to advance our understanding of the mating biology and reproductive success of the mosquito vector *Aedes aegypti*. Then he desired to pursue a second M.S. degree at Cornell under the direction of Dr. Harrington in the fall of 2009.

To my parents, my family with all my love for their guidance and inspiration

ACKNOWLEDGMENTS

Many people have made vital contributions to my life and education during my time at Cornell. I am forever indebted to them for all they have provided me.

I would like to thank to my Special Committee Chair, Dr. Laura Harrington. She took a huge risk on me and simply provided me with the chance when others might not have. I will never forget the opportunity she afforded me. She spent her tight schedule to meet and guide me at least once a week for 4 years. I will carry her scientific ideology with me throughout my career. The "hands off" approach proved invaluable for my education, teaching self reliance and independent work. I also would like to thank members of my Special Committee, Dr. Mariana Wolfner and Dr. Angela Douglas for guidance and valuable suggestions. I greatly appreciate to Dr. Laura Sirot and Dr. Michelle Helinski for supporting throughout my researches. Appreciation is extended to Dr. Alongkot Ponlawat (Department of Entomology, AFRIMS, Thailand) for providing some field work equipments, Dr. Anawat Suwanagul (Thailand Institute of Scientific and Technological Research, Thailand) and people in his lab for proving facilities and equipments in his lab to conduct my field research. I appreciate the expert statistical advice of Francoise Vermeylen.

I greatly appreciate all field help from my spouse, Dr. Rujira Deewatthanawong and all members in her family for all field site.

Thanks belong to Sylvie Pitcher and all members of Harrington lab for friendship, personal support. Thanks to many friends have been consistently supportive throughout my time in graduate school. I am particularly grateful to Kris Corda, a friend in the truest sense. Many thanks for Jiahe Li, Jeerapond Leelawattanachai and all Thai friends and friends at Cornell for

whenever I needed help whether it was about the lab work or simply to escape from the rigors of life. I appreciate all administrative and personal support during pursuing my M.S. degree at Cornell.

Finally, I am indebted to my parents, my wife, my sisters, my brothers, and my whole family for their love and support.

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CHAPTER ONE

INTRODUCTION

Dengue and dengue vectors

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most important arthropod-borne viral infections in humans. An estimated 2.5-3 billion people are at risk of infection globally (Gubler 2002, Remme et al. 2002, WHO 2006).

Dengue viruses are primarily transmitted by the *Aedes aegypti* mosquito which inhabits tropical and subtropical regions of the world (Gubler 2002). Adult *Ae. aegypti* mosquitoes prefer to rest indoors, and domestic day-biting females preferentially feed on humans (CDC 2005, Harrington et al. 2001, Scott et al. 1993, Scott et al. 2000). In addition, this species utilizes indoor and peridomestic breeding sites including artificial water storage containers. The species can be passively dispersed around the world in containers via human trade and travel. Rapid increases in human populations, travel, urbanization and climate change can create more favorable habitats for mosquitoes resulting in an expanded range (CDC 2005, Gubler 2002, Vezzani 2007). Vertebrate blood is essential for mosquito ovarian development and oviposition (Harrington et al. 2001). Dengue transmission occurs when an *Ae. aegypti* female consumes blood-meals from viremic hosts. The human viremic window usually last for 3 to 14 days (average 4 to 7 days) (Gubler 1998). Viral particles infect cells lining the midgut of the mosquito and progressively infect every organ, eventually reaching the salivary glands. After an extrinsic incubation period of 8-12 days in the mosquito, dengue viruses are transmitted to another person when injected in the saliva of a blood feeding mosquito (Gubler 1998). Once infected with dengue virus, the female will remain viremic throughout her entire life (Blair et al. 2000).

Dengue virus belongs to genus *Flavivirus*, family Flaviviridae (Westaway and Blok 1997). There are four closely related but distinct serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). When humans are infected with one serotype, the infection provides lasting immunity against that serotype. However, there is no cross-protective immunity in humans for other viral serotypes. Hence, an individual living in highly dengue endemic areas can have as many as four infections during their lifetime (Gubler 1998, Mackenzie et al. 2004).

Two forms of dengue symptoms dengue fever (DF) and dengue hemorrhagic fever (DHF) occur in humans. Symptoms of classical DF include sudden onset of flu-like symptoms including fever, headache, muscle aches, pain behind the eyes, and rash (Gubler 1998). DHF is a more severe and potentially deadly form. Symptoms of DHF are high fever, abdominal pain, vomiting, intense capillary leakage, and platelet count of less than 100,000 per mm^3 (Nimmannitya 1997, Igarashi 1997). Shock and death may occur in severe cases. DHF is often associated with the secondary infection by other DEN serotypes, viral virulence, and epidemiological factors (Halstead 1997, Gubler 1998).

Current control strategies

Currently, many mosquito control strategies are used for these vectors including chemical insecticides, biological control, and environmental management. These methods are costly and require public education and community involvement. The main stumbling blocks to reduce or eradicate these vectors, in addition to effective control programs are public education and community participation (Hemingway and Ranson 2005, Opiyo et al. 2007).

To date, the most effective and cheapest way to combat dengue is use of insecticides. Most insecticides are developed for use on agricultural insects. These agrochemicals are

eventually formulated for use in public health (Hemingway and Ranson 2005). Agricultural use can lead to indirect exposure of disease vectors and resistance development. Many examples of this phenomenon are described by Hemingway and Ranson (2005), Chandre et al. (1999) and Ranson et al. (2000).

Since the 1900s, biological control strategies have been employed to target disease vectors such as use of predators, pathogens, parasites, or microbial toxins (Hemingway 2005). Mosquito fish (*Gambusia affinis*) were first successfully used to control larvae in many countries. In the early 1940's, a highly effective broad-spectrum insecticide (Dichlorodi-phenyltrichloroethane, DDT) was used for mosquito control. Eventually DDT was banned due to concerns over safety, environmental effects and resistance development in mosquitoes including in *Ae. aegypti* (Neely 1964, Bang et al. 1969, Chapin and Wasserstrom 1981). In the early 1960's, there were many concerns about the environmental impact of chemical pesticides which placed more emphasis on biological control of mosquitoes. The entomopathogenic nematode (*Romanomermis culicivorax*) and the protozoan (*Nosema algerae*) were investigated for control of mosquito larvae. However, these approaches for mosquito control in nature were unsuccessful because the agents were costly to produce and were subject to rapid environmental degradation (Chapman 1974, Kaya and Gaugler 1993, Legner 1995). Recently, the most effective biological mosquito control agent is *Bacillus thuringiensis israelensis*. This bacterial species produces a toxin that is highly specific to mosquito larvae. Less expensive production and formulation technologies make these bacterial products practical for widespread use in many regions (Backer and Ascher 1998, Hemingway 2005). Using sterile males as a competitor of wild-type males is another non-insecticidal mosquito control technique. Successful sterile male release programs such as that for screwworm eradication in North and Central America (Wyss

2000) has raised enthusiasm for this approach to control mosquitoes. However, early sterile male releases with mosquitoes were not successful due to a variety of factors including reduced competition of sterile males with their wild type counterparts, low production capacity, high rates of immigration/emigration, as inefficient sexing techniques, and insufficient knowledge of mosquito mating behavior (Wood 2005). Recently, control strategies that aim to use genetic approaches to sterilize males or reduce population levels have gained more attention for mosquito control including with *Ae. aegypti* (James 2007, Fu et al. 2010).

Mosquito mating biology

Understanding the mosquito mating system is essential for a greater understanding of mosquito behavior, evolution, genetics, and population structure. In addition, insights into the mating system may reveal new targets for vector control strategies and the elimination of mosquito-borne diseases.

Ae. aegypti males are considered to be polygynous, potentially mating with many females within a day. A period of 18-24 h post-emergence is generally required for the completion of sexual maturation, which in males includes a 180° rotation of the genitalia (Clements 1999). In nature, *Ae. aegypti* mating commonly occurs near hosts. Males appear around their hosts to encounter females (Cator et al. 2011). The male utilizes acoustic cues at short range to identify conspecifics and then rapidly grasps the female to initiate copulation. The average duration of copulation ranges from 6-20 s for *Ae. aegypti* (Helinski and Harrington 2011). During male ejaculation, semen is deposited in female bursa. Sperm and seminal fluid proteins move into the bursa inseminalis and eventually to the spermathecae for storage.

Ae. aegypti females have be considered to be monandrous over their lifetime (Craig 1967, Spielman et al. 1967, Clements 1999). Though results from several laboratory studies indicate that females sometime mate multiple times. Gwadz and Craig (1970) observed multiple mating at a low frequency (i.e., 7.5 %) when *Ae. aegypti* females were exposed simultaneously to males of several mutant genotypes (e.g., MISS-MARK, COLORLESS, and BULBOUS). In a recent study of *Ae. aegypti* conducted in field cages in Mexico using stable isotope semen-labeled males (^{15}N or ^{13}C), Helinski et al. (2012) reported that up to14% of females had both labels in the spermathecae, indicating they received semen for more than one male.

Many female mosquito species mate before consuming the first blood meal, but a large proportion of virgin *Anopheles* blood-feed prior to mating and a blood meal is essential for the development of a metabolic energy reservoir (Lyimo and Takken 1993, Takken et al. 1998). Some females such as crab-hole mosquito (*Deinocerites cancer* Theobald) are inseminated immediately as they emerge from the pupal cases; males wait next to the emergence site to grasp females shortly after emergence (Provost and Haeger 1967). Plant nectar or other carbohydrate sources may be required to provide an energy reservoir to males and females for flying and mate finding (Foster 1995, Foster and Takken 2004).

Mosquito body size and reproductive success

Body size is a well-documented factor influencing mating and reproductive success for both males and females in many vertebrate and invertebrate animals (reviewed in Crespi 1989). Ponlawat and Harrington (2007) found that larger male *Ae. aegypti* had greater spermatozoa numbers. They also transferred more sperm to females (Polawat and Harrington 2009). Helinski and Harrington (2011) also demonstrated that large male *Ae. aegypti* had greater mating capacity

than small males in rapid sequential mating. Body size can also influence ecological traits including adult mosquito dispersal. The advantage of body size is also well known for female mosquitoes, where body size is proportional to the number of gametes and fecundity (Steinwascher 1982, Washburn et al. 1989, Briegel 1990 Lyimo and Takken 1993, Renshaw 1994). Laboratory and field studies showed that in several mosquito species such as *Ae. triseriatus* (Say), *Ae. punctor* (Kirby) and *Mansonia dyari*, an increase in survival rate is positively correlated with body size (Haramis 1985, Packer and Corbet, 1989, Lounibos et al. 1990), but others failed to demonstrate such a relationship for *Ae. albopictus* (Skuse) (Mori 1979) and *Ae. sierrensis* (Ludlow) (Washburn et al. 1989).

Little information is available regarding competitiveness and reproductive success of male mosquitoes based on their body size. Many factors could potentially influence male reproductive success including age, body size, sperm quantity, and sperm quality. Previous studies reported an age related effect on male mating capacity as measured by the presence of spermatozoa in cohabited females. For example, the peak of *An. culicifacies* Giles male mating behavior occurred when males were 5-7 day-old, and mating ability was reduced for males 10-12 day-old (Mahmood and Reisen 1994). Okanda et al. (2002) reported that male *An. gambiae* held in laboratory cages preferentially mated with large females. In *Ae. aegypti* studies, Hausermann and Nijhout (1975) demonstrated an influence of male age of *Ae. aegypti* (L.) on successful mating by comparing morphological traits of testes and seminal vesicles of 0-30-day-old males. Beyond basic descriptions of mating biology, the factors influencing mosquito mating success in mosquitoes from dengue-endemic areas have rarely been studied.

Sperm capacity and depletion

A male's reproductive success is only limited by the number of mates he can encounter and inseminate during his lifetime (Bateman 1948) and the total number of viable offspring that females can produce. Males copulating with different females in rapid succession often suffer from sperm depletion (Perston et al. 2001, Wedell and Ritchie 2004). Previous studies in Australian blowfly (*Lucilia cuprina*) and red flour beetle (*Tribolium castaneum*) demonstrated that the proportion of sperm in females decreased with mate succession (Smith et al. 1990, Qazi et al. 1996). Early studies in *Ae. aegypti* males indicated that a single male is capable of inseminating 4-6 females sequentially (Gwadz and Craig 1970, Jones 1973, Foster and Lea 1975). Recent laboratory studies by Helinski and Harrington (2012) demonstrated that large and small males become depleted when sequentially mated with up to five females for large males and three females for small males over an 8 hour period. In that study fecundity was reduced by 50% for females that had mated with depleted males.

Role of seminal fluid proteins (sfps) in *Ae. aegypti*

Insemination of female insects introduces not only gametes, but also substances (seminal fluids) produced by the male accessory glands and ejaculatory duct. During copulation, semen is deposited in the female bursa seminalis, primarily as medium for sperm to move into the opening of the spermathecal ducts. Early studies by Craig (1967) demonstrated that a substance (called "matrone") derived from male accessory glands and artificially administered to virgin females (via injection or transplantation) prevented them from mating. These seminal products were partially purified. Fuchs and others (1968) reported that "matrone" consisted of at least two separate proteins namely α and β and both proteins were essential to induce mating inhibition (Fuchs 1969, Fuchs and Hiss 1970). Hiss and Fuchs (1972) demonstrated that α protein alone is sufficient to stimulate oviposition in females (Hiss and Fuchs 1972). Leahy and Craig (1965),

Ramalingam and Craig (1976) showed that virgin females receiving an implant of male accessory glands were stimulated to oviposit and became sexually refractory (Fuchs et al. 1969, Fuchs and Hiss 1970). In addition, AG homogenates increased ovarian development (Feyvogel et al. 1968, Klowden and Chambers 1991, 1992). Judson (1967) also demonstrated that a similar feeding pattern as mated females could be established in virgin females by implanting a male accessory gland. Furthermore, Edman (1970) noted that older and unmated females digested blood meal than younger and mated females. AG substances may be absorbed into the hemolymph and potentially can contribute to the nutritional state of females, affecting physiology and influencing a variety of effects on female behavior, include enhancing their reproductive success (reviewed in Clements 1999, Klowden 1999, Friedel and Gillott 1977, Giesel et al. 1989, Raina 1989).

Recently, proteomics and bioinformatic approaches have been adapted to identify Sfps in many mammals and insects. Identification of Sfps and their transfer in *Ae. aegypti* has recently been published (Sirot et al. 2008, Sirot et al. 2011). The authors identified 93 male-derived Sfps transferred to females during mating. They also discovered several predicted membrane-bound and intracellular proteins in the seminal fluids are transferred to females, supporting the hypothesis that *Ae. aegypti* Sfps are released from the accessory gland cells through apocrine secretion, as occurs in mammals. Functional analyses to identify the role of individual Sfps in mated females are underway.

There were two main objectives of my research. One was to develop an AG-specific promoter construct for male *Ae. aegypti* that could be used in future studies to target the tissue, or to target RNAi for seminal fluid protein genes. This was part of a larger project to develop novel mechanisms for genetic mosquito control. Another objective was to investigate some

fundamental aspects of mating biology in this species, such as sperm allocation patterns as influenced by female reproductive quality and mating preferences in semi-field conditions in Thailand. Collectively, this work was intended to contribute to a greater understanding of mosquito mating biology as it relates to the use of transgenic males for population control.

CHAPTER TWO

DEVELOPMENT OF A MALE ACCESSORY GLAND SPECIFIC PROMOTER FOR *AEDES AEGYPTI*

INTRODUCTION

There is an urgent need to develop novel strategies for mosquito vector control. One type of strategy involves genetic approaches that target mosquito reproduction. For example, one could knock down or knock out male seminal fluid protein genes that have the potential to play important roles in female fecundity or as a way to ensure monogamy (Sirot et al. 2011).

The goal of this study was to develop a promoter construct for one of *Ae. aegypti* male seminal fluid proteins (sfps), AAEE010824 - a protein that is transferred to females during mating (Sirot et al. 2008)- as the first step towards functional analysis of sfps in female mosquitoes. These proteins have been shown to influence feeding and reproductive behaviors in mosquitoes as described in Chapter 1. Our ultimate goal is to target sfp genes as a novel mechanism for genetic control of female mosquito feeding behavior and biology.

Here, I report on the identification of a promoter region of AAEL010824 gene construct of an EGFP reporter fusion. The promoter will be a useful tool for future transgenesis studies of predicting function of seminal fluid proteins in *Ae. aegypti*.

MATERIALS AND METHODS

Determining the full-length *c*DNA of AAEL010824

Mosquitoes. A Liverpool strain of *Ae. aegypti* was obtained from Notre Dame University. This colony has been maintained in the laboratory for more than 40 years. Mosquitoes were reared in an environmental chamber set with a temperature of 22-30 ^{o}C, a photoperiod 14 hrs light: 10 hrs dark, and 80 % RH. Eggs were hatched in a 0.5 L flask under a vacuum for 30 min. A small amount of *Aedes* diet (30 µg, 1:1 ratio of lactalbumin/brewer's yeast) was added to 200 ml water. Larvae were held in the flask for 24 hrs at 28 ^{o}C until they were large enough to be sorted into rearing trays. Larvae were reared following the methods described by Ponlawat and Harrington (2007). Pupae were separated individually into plastic vials. Emerged adults were sorted by sex and maintained in 5 L plastic cages with a 20% sucrose solution until experiments commenced. Six hundred virgin 3-5 day-old males were selected and accessory glands were dissected for RNA isolation.

RNA extraction. Male accessory glands were dissected on an RNase free glass slide over ice with 20 µl of RNase free phosphate-buffered saline (PBS). Accessory glands were torn gently with fine forceps under a stereo microscope and pooled in a 1.5 ml microcentrifuge tube containing 300 µl Trizol (Invitrogen® Carlsbad CA, USA) and RNA was isolated following the manufacture's protocol. RNA purity was confirmed using a NanoDrop™ spectrophotometer model ND-2000 (Thermo Scientific) and 1% agarose gel electrophoresis.

Rapid Amplification of cDNA Ends (RACE). Rapid amplification of cDNA ends (RACE) was performed using the GeneRacer™ system (Invitrogen® Carlsbad CA, USA) with SuperScript™ III, following the manufacturer's instructions, using 1 µg of RNA per reaction. An oligo dT primer was used in 3´ends of the cDNA and three individual (oligo dT, 5´cDNAsynth_GSP, and random (N6)) primers were used in 5´ends of the cDNA. Primers are shown in Table 1. The 3´and 5´ of cDNA ends were diluted 10 times and 1 µl was used as a PCR template. Primer GeneRacer™ 3´ and 3´RACE-GSPF_1 were used in 3´ends PCR (Table 1). Primer GeneRacer™ 5´ and 5´RACE_GPSR_3 (Table 1) were used as 5´ends PCR. Approximately 0.2 and 1 kb DNA fragments were amplified for 3´cDNA (Figure 1) and ~0.2 kb of DNA fragment was amplified in three synthesized types of 5´ cDNAs (Figure 2). PCR products of 3´ and 5´ends were cloned into pCR4-TOPO vector (Invitrogen® Carlsbad CA, USA) and transformed into TOP10 *Escherichia coli* competent cell (Invitrogen® Carlsbad CA, USA) following the manufacturer's guidelines.

Screening 3´ and 5´ RACE clones. Thirty six individual colonies from 5´and 3´ clones were randomly selected and used as PCR templates. PCR was performed using Gotaq polymerase (Promega® Madison WI, USA) following manufacture's procedure with some modifications and 0.6 µl of each 10 µM T7 and T3 primers (Table 1). PCR conditions were as follows: initial denaturation at 95 ^{o}C for 2 min, followed by 35 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 50 ^{o}C for 30 sec, and extension at 72 ^{o}C for 90 sec. PCR products were visualized on a 1% agarose gel (Figure 1B and 2). Plasmids were extracted using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen® Carlsbad CA, USA) following the manufacturer's guideline. Plasmids were sequenced by the Cornell University Life Sciences Core Laboratories Center. Sequencing data were analyzed to determine 3´ and 5´ UTR of AAEL010824 gene.

Table 1. Primer sequences used for amplification 3´ and 5´ RACE of AAEL010824 gene

Primer Name	Sequence (5´- 3´)	Purpose
3´ RACE		
Oligo dT	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$	3´cDNA synthesis
GeneRacer™ 3´	GCTGTCAACGATACGCTACGTAACG	3´ PCR specific amplification
3´RACE-GSPF_1	TGGCGACATGTGGGTCATTACCAGAA	3´ PCR specific amplification
5´ RACE		
Oligo dT	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$	5´cDNA synthesis
5´cDNAsynth_GSP	TCGGAGCCCCTTATGTAGACGTA	5´cDNA synthesis
GeneRacer™ 5′	CGACTGGAGCACGAGGACACTGA	5´ PCR specific amplification
5´RACE_GPSR_3	ACGCGACTTTCGCACGGACA	5´ PCR specific amplification
3´ and 5´ RACE control		
Control Primer A	GCTCACCATGGATGATGATATCGC	3´ PCR control amplification
Control Primer B.1	GACCTGGCCGTCAGGCAGCTCG	5´ PCR control amplification
T7	TAATACGACTCACTATAGGG	Screening 3´and 5´ RACE clones
T3	ATTAACCCTCACTAAAGGGA	Screening 3´and 5´ RACE clones
M13Forward	GTAAAACGACGGCCAG	Sequencing
M13Reverse	CAGGAAACAGCTATGAC	Sequencing
10824-ChR1	CGGTCCCCGTATGCAACCAC	PCR confirming 5´ ends RACE
10824-ChR2	AGCCATCCCTAGGCGGAACA	PCR confirming 5´ ends RACE
10824-ChR3	CGCTCAGTACAGCGTCGGACA	PCR confirming 5´ ends RACE
10824-ChR4	CACAAGAACTGGCTGGCTCTTAAACG	PCR confirming 5´ ends RACE
GeneRacer™ 5′	CGACTGGAGCACGAGGACACTGA	PCR confirming 5´ ends RACE

Confirming 5´ RACE results using PCR. 5´ cDNA end synthesized using oligo dT was used as a PCR template using Hi-Fi Taq polymerase (Invitrogen® Carlsbad CA, USA) following manufacturer's protocol. Four primer sets (Table 1) were used in reactions to confirm the 5´ end RACE results. Results and primer design are shown in Figure 3.

AAEL010824 promoter construct of *Ae. aegypti* THAI strain

Mosquito. *Ae. aegypti* Thai strain was established from mosquitoes collected in Soi Lat Krabang 36, Bangkok (15°7193′ N, 101°752′ E), Thailand from May-June 2009 and had been maintained in our laboratory since August 2009 in an environmental chamber with the same conditions as described above.

DNA isolation. DNA was isolated from two virgin males using Pure Gene Tissue Core Kit B (Qiagen-Gentra Minneapolis MN, USA) with the following modifications from company's guidelines. Briefly, a fresh mosquito was homogenized in 100 μl cell lysis solution, and incubated at 65 ^{o}C for 10 min. Then, 33μl protein precipitation solution was added, mixed, incubated on ice for 5 min, and centrifuged at 13,000 rpm for 3 min. Supernatant was transferred to new tubes, and 100 μl of 100% isopropanol was added and incubated at -20^{o}C for 10 min. Tubes were centrifuged at 13,000 rpm for 20 min. The DNA pellet was washed twice with 500 μl of 70% ethanol. The pellet was dried in at room temperature for 10 min and dissolved with 20 μl of hydration solution from the kit.

Requested constructs. Two constructs were received from R. Harrell at the Insect Transformation Facility at the University of Maryland Institute for Bioscience and Biotechnology Research. pBac[3xP3-DsRedaf] was constructed by cloning a 1.3 kb *Eco*RI (Blunted with Klenow)/*Nru*I fragment from pSL-3xP3-DsRedaf into p3E1.2(Cary et al. 1989) cut with *Bgl*II (Blunted with Klenow)/*Hpa*I. The *Eco*RI, *Nru*I, *Hpa*I, and *Bgl*II sites were destroyed (Horn et al 2002, Horn et al. 2003). The second construct, pBac[3xP3-EGFPafm], was created by cloning the 0.1 kb *Fse*I/*Bgl*II fragment from pSLfa1180fa into pBac[3xP3-EGFPaf] cut with *Fse*I and *Bgl*II, thereby removing 800 bp of the transposase coding region (Horn and

Wimmer 2000, Horn et al. 2000). The EGFP region in this construct served as a reporter for the AALE010824 promoter.

PCR amplification and cloning of AAEL010824 promoter region. Full length cDNA sequences were blasted against *Ae. aegypti* genome in www.vectorbase.org and NCBI. Sequence information beginning approximately 5 kb upstream of the transcription start site (AUG) of AAEL010824 were selected from *Ae. aegypti* in vectorbase. NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/) and Promoter 2.0 Prediction Server (CBS, Technical University of Denmark DTU, http://www.cbs.dtu.dk/index.shtml) were used to estimate a predicted promoter region of this gene. Predicted 5´ UTR of AAEL010824 promoter is shown in the Table 2.

Table 2. Predicted 5´ UTR upstream AAEL010824 promoter results using Promoter 2.0 Prediction Server (CBS, Technical University of Denmark DTU)

5´ UTR upstream position (Kb)	Score	Likelihood
3.6	1.082	Highly likely prediction
4.4	0.698	Marginal prediction

5´ UTR upstream (~ 5 kb) region was amplified using iProof high fidelity DNA polymerase (Bio-Rad® Hercules CA, USA) following manufacturer's protocol with slight modifications. Briefly, 1 µl of diluted 50x gDNA was added into 20 µl PCR reaction containing 4 µl 5XiProof HF buffer, 0.4 µl 10 mM dNTPs, 0.6 µl of each 10 µM 10824(5K)-*Fse*I-F and 10824(5K)-*Fse*I-R primers (Table 3), 13.2 µl water, and 0.2 µl iProof DNA polymerase (2U/µl).

Table 3. Primer sequences used for AAEL010824 promoter construct

Primer Name	Sequence (5´- 3´)	Purpose
10824(5K)-*Fse*I-F	TAATAGGCCGGCCCTGGGCTCGTTAATCTCGAA	PCR amplification 5´ UTR of AAEL010824
10824(5K)-*Fse*I-R	TAATAGGCCGGCCGAATAACGGATGTCACAAGAACTGGCTG	PCR amplification 5´ UTR of AAEL010824
*Fse*I-EGFP-F_5	TAATAGGCCGGCCCCACCATGGTGAGCAAGGG	PCR amplification of EGFP
*Asc*I-EGFP-R	TAATAGGCGCGCCGTACGCGTATCGATAAGCTTAA	PCR amplification of EGFP
DsRed-CF_1	CCCGGCTACTACTACGTGGA	Screening clone constructs
DsRed-CR_1	TCCTCTCTGCTCTTCTGCAA	Screening clone constructs
10824-TXL-R_1	TGCCGGGACCACTAGTTTTA	Screening clone constructs
EGFP-R_1	TGCTCAGGTAGTGGTTGTCG	Screening clone constructs
10824-TXL-F_1	GTTCTGGAACAACGTCACGA	Screening clone constructs
M13Forward	GTAAAACGACGGCCAG	Screening clone constructs

The reaction was subjected to denaturation at 98 ^{o}C for 30 sec and 35 cycles of denaturation at 98 ^{o}C for 10 sec, annealing at 72 ^{o}C for 10 sec, and extension at 72 ^{o}C for 72 sec (extension time was approximately 15s per 1 kb of target), followed by the final extension at 72 ^{o}C for 10 min. Products were visualized on 1 % agarose gels. An approximately 5 kb band was excised under low UV light. The DNA fragment was purified by using QIAquick Gel Extraction Kit (Qiagen® Valencia CA, USA) following the manufacturer's instructions. A-overhangs were added into 5´and 3´end of fragments using Hi-Fi Taq polymerase (Invitrogen® Carlsbad CA, USA). Briefly, 8.7 μl purified product was added into a tube containing 1 μl 10X Hi-Fi Taq buffer, 0.2 μl 2.5mM dATP, and 0.1 μl Hi-Fi Taq polymerase. Reaction tubes were incubated at 72 ^{o}C for 15 min and cooled on ice. Product was immediately cloned into pCR®-XLTOPO vector and transformed into chemically competent TOP10 *E. coli* cells (Invitrogen® Carlsbad CA, USA) following the manufacturer's guidelines. Cloned colonies were randomly selected and used as PCR templates. PCR colony screen was performed using the method described above with M13

forward and 10824-TXL-R_1 primers (Table 3). A plasmid containing the AAEL010824 promoter was prepared using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen® Carlsbad CA, USA) and sequenced at Cornell University Life Sciences Core Laboratories Center. To confirm the promoter sequences, sequencing data were blasted with the sequence information from *Aedes* vectorbase genome using NCBI Blasts.

Cloning pBac[3xP3-EGFP-DsRedaf] construct. pBac[3xP3-EGFP-DsRedaf] was constructed by amplification of EGFP plus SV40 fragment (995bp) from pBac[3xP3-EGFPafm] plasmid using iProof high fidelity DNA polymerase (Bio-Rad, USA) according to manufacturer's guideline. One µl of diluted 1000x of plasmid was used as a PCR template. *Fse*I-EGFP-F_5 and *Asc*I-EGFP-R primers were used in the PCR reaction (Table 3). PCR products were purified using QIAquick PCR Purification Kit (Qiagen® Valencia CA, USA) and ligated into a digested *Fse*I/*Asc*I backbone pBac[3xP3-DsRedaf] using T4 ligase (Promega® Madison WI, USA) following the protocol guidelines. Ligated vector was transformed into TOP10 *E. coli* competent cells (Invitrogen® Carlsbad CA, USA). Thirty six colonies were randomly selected and PCR was used to screen positive clones following the method described above, with the exception that DsRed-CF_1 and *Fse*I-EGFP-F_5 primers (Table 3) were used in these reactions. PCR results are shown in Figure 5A. Finalized DNA plasmid with PureLink™ Quick Plasmid Miniprep Kit (Invitrogen® Carlsbad CA, USA) and cloned constructs were confirmed by digesting with *Sac*II and *Fse*I. Digested plasmid reactions were visualized on a 1 % agarose gel. The correct clone of approximately 1,976 and 7,408 bp fragments was detected as shown in Figure 5B. The plasmid was sequenced by the Cornell University Life Sciences Core Laboratories Center. Sequencing data were blasted with sequence from constructs to confirm the orientation of EGFP.

pBac[3xP3-AAEL010824EGFP-DsRedaf] final construct. To generate pBac[3xP3-AAEL010824EGFP-DsRedaf] construct, 1μl of pBac[3xP3- EGFP-DsRedaf] (backbone) and pCR®-XLTOPO-AAEL010824 (insert) were digested with *Fse*I (NEB® Ipswich MA, USA) following manufacturer's directions. Digested backbone was dephosphorylated at 5´ phosphate groups using calf intestinal phosphatase (CIP) (NEB® Ipswich MA, USA). Dephosphorylated products were purified using QIAquick PCR Purification Kit (Qiagen® Valencia CA, USA) as described in company's guidelines. Digested backbone and AAEL010824 promoter were ligated using T4 ligase (Promega® Madison WI, USA) and ligated product was transformed into TOP10 *E. coli* competent cells (Invitrogen® Carlsbad CA, USA). Three hundred clones were picked and streaked into a new prewarmed LB plus100μg/mL amplicilin plate and incubated at 37 ^{o}C overnight. After 24 hrs, colonies were screened for positive transformation using colony PCR as described above with DsRed-CR_1 and 10824-TXL-R_1 primers (Table 3). PCR products were visualized on a 1% argarose gel (Figure 6A). Eight colonies containing the 756 bp amplicon were confirmed by a second set of PCR using EGFP-R_1 and 10824 TXL F_1 primers (Table 3). The DNA fragment (~1,080 bp) was amplified and PCR products are shown in Figure 6B. Orientation of an insert from plasmids of 4 positive colonies was confirmed by *Sac*II and *Xho*I digestions. Fragments of approximately 6.16, 4.024, and 2.27 Kb were visualized in 1% agrarose gel as shown in Figure 6C. Plasmid construct number 11 was submitted for sequencing at Cornell University Life Sciences Core Laboratories Center. Orientation of AAEL010824 and the cloning construct were confirmed. Final plasmid constructs were shipped to the Insect Transformation Facility.

RESULTS

Determination of the full-length of AAEL010824 using RACE

Using GeneRacer™ system approach, full-length AAEL010824 gene sequences of 695 bp were identified with 22 bp (64.3%) of 5´ UTR and 48 bp (69.3%) of 3´ UTR. Sequence data of the 5´ and 3´ UTRs are shown in Table 4 and Figure 4. PCR products are shown in Figures 1 and 2. In addition, another less variant type of 3´UTR (15.4%) containing 726 bp (Figure 1, 4 and Table 4) was found.

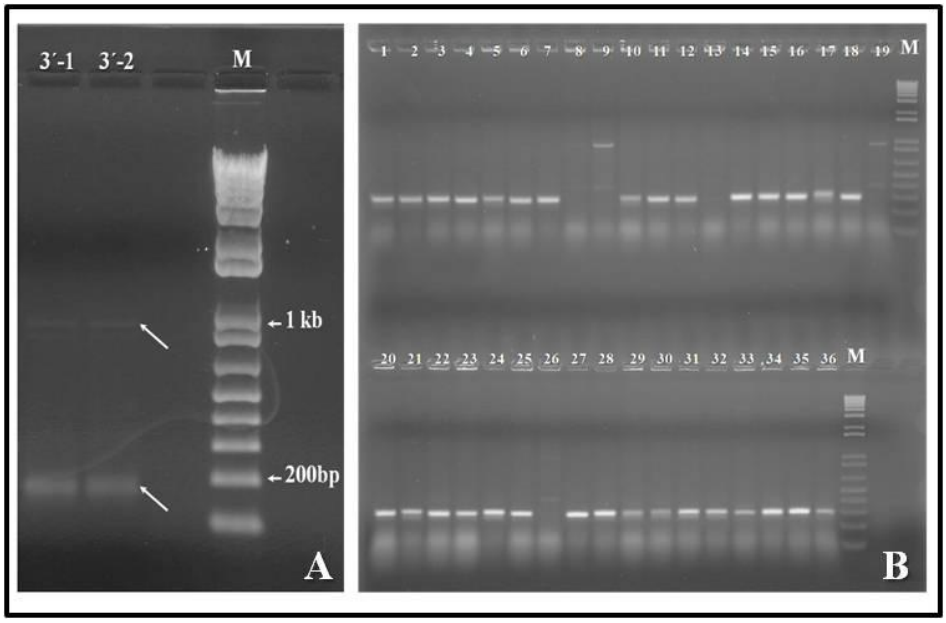


Figure 1. PCR amplification of 3´-end of AAEL010824 from cDNA. (**A**) two band of approximately 200 and 1,000 bp amplicons of 3´-end were visualized on a 1% agarose gel (white arrows). 3´-1 and 3´-2 are a micro-liter of non diluted and 10 x diluted 3´-end cDNA templates in PCR reaction, respectively. M is a 1Kb DNA molecular weight marker (Invitrogen® Grand Island, NY. USA). (**B**) Thirty six clones were screened for 3´-end insertion. Colonies number 1, 2, 3, 4, 9, 11, 15, and 19 were sent for sequencing for 3´-end results (numbers indicate colony numbers).

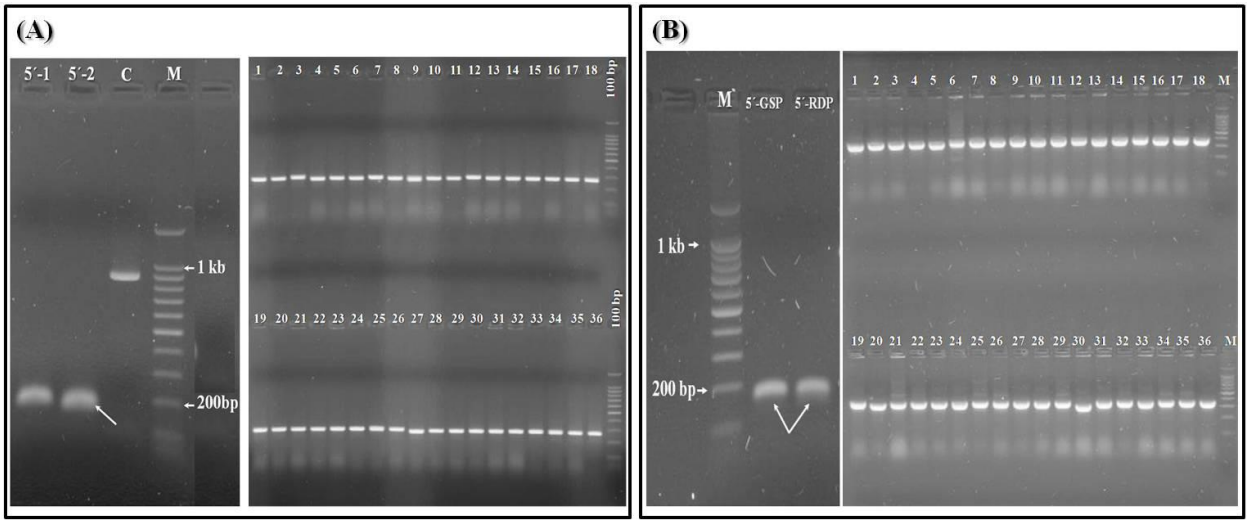


Figure 2. PCR amplification of 5´-end of AAEL010824 from cDNA. (**A**) 5´-end of AAEL010824 cDNA was synthesized using oligo dT and an approximately 200 bp amplicon was amplified with PCR (white arrow) [5´-1 and 5´-2 are 1µl of non diluted cDNA and 10x diluted cDNA, C: HeLa RT template (control PCR reaction from GeneRacer Kit, Invitrogen®), M is a 100 bp DNA molecular weight marker (Invitrogen®), thirty six clones were screened for 5´-end insertion using colony PCR. (**B**) 5´-end of AAEL010824 cDNA was synthesized using the gene specific primer (5´-GSP) and the random primer (5´-RDP); 1 µl of 10x diluted cDNA was used in PCR (white arrows are 5´-UTR amplicons). Thirty six clones were screened for 5´-end insertion using colony PCR.

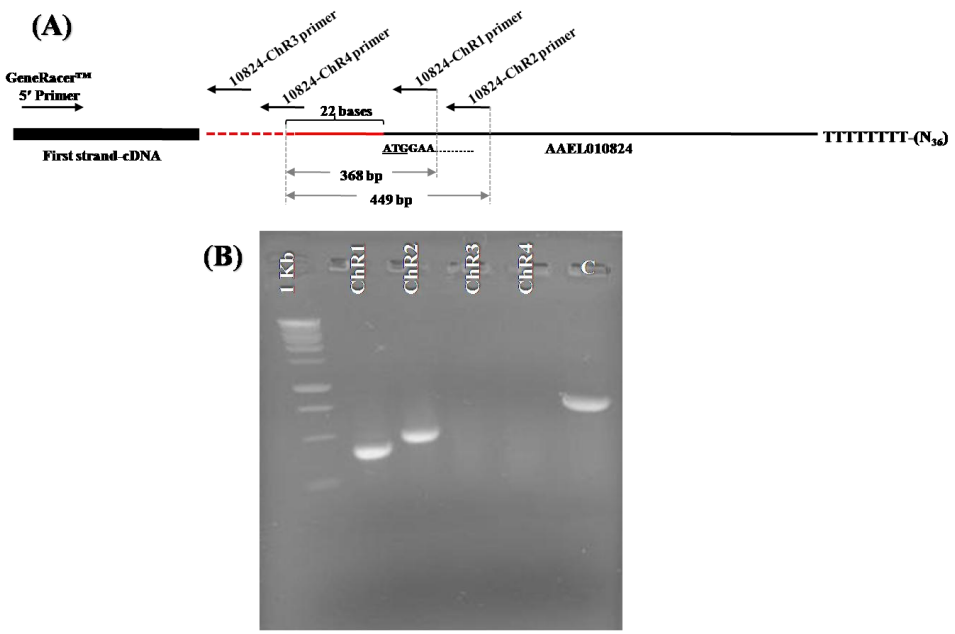
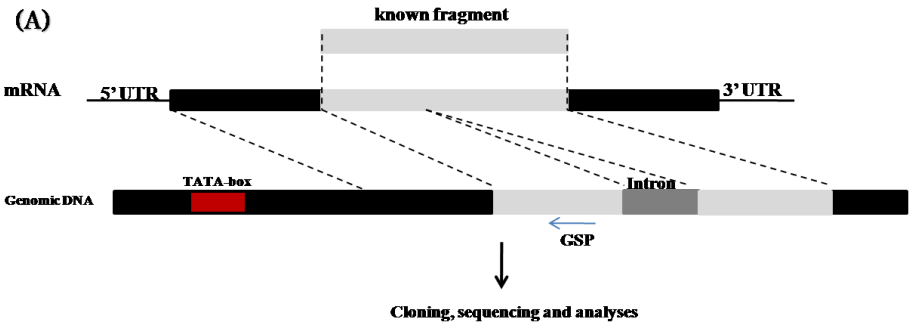


Figure 3. (A) Schematic diagram of primer design for checking 5´UTR results of AAEL010824 gene. (**B)** Four PCR reactions with different reverse primers and GeneRacer™5´primer (forward). Lane 1: 1 kb DNA marker (Promega® Madison, WI, U.S.A.), lane 2: 10824-ChR1 reverse primer, lane 3: 10824-ChR2 reverse primer, lane 4: 10824-ChR3 reverse primer, lane 5:10824-ChR4 reverse primer, and lane 6 or C: control PCR reaction using HeLa RT template with Control Primer B.1 (reverse) and GeneRacer™ 5´ primer (forward) primer from the GeneRacer Kit (Invitrogen® Carlsbad, California, USA). Gene specific primers were designed based on AAEL010824 sequences from vectorbase.org and GENE Racer™ kit (Invitrogen®) for the primer condition using Primer 3 program (http://frodo.wi.mit.edu/primer3/).



(B) The most common variant: 5' UTR in red, most common 3' UTR in blue, coding sequence (beginning and end) in black

CTTGTGACATCCGTTATTCAAAATGGAACTTCTA.....CAAGCTGGGTAAGTGTTAGTTCATAGACAATGGCGTTTAAGCGAATAAAATAT
CGATTTCCCCTTGT

The less common 3' variant: 5' UTR in red, less common 3' UTR in green, coding sequence (beginning and end) in black

CTTGTGACATCCGTTATTCAAAATGGAACTTCTA.....CAAGCTGGGTAGGGTAGTGGCCCACTAGTAGTGGCTACGGTTAGGATAAGGCA
GAGCCAATCAGATTAGTATAGAAGAACAGCACAACCATGCGGACTCATACAAAATGTCAGCCCAAGTTACGCTAGTTCTAGAACCTTACTTTT
GTAAGGGGACCTTTTGTATACATAATCTCGTTTATCCAGTTTTTGTTGTTTTCAGTGAACCAGGAATGGCAAACTCACGTGCAATGTCTCGTTT
CAAACGATTTGTCGTATACTGCGTTTCAAAAGTCTTTCCACTTACTGTATTATTGCTAATGCTCAACAATGACATTATCTAGGCCAGCTCAGTT
ATCAGTTTGAGAGGCTCCAGTCCTAGATAAATGGATTAGTGAATAAAGGCAATCGCCTAAACCTCATGGTATCTGCTAGAAGAAGTGTTAGAC
ATAAACCTCAGCTCGAACAGAACTAGTCACGAGTTAAGGAATCATTATCTGACCATCGCTACATCTTCTTTTGACACTTGAATGTAATTTTGCA
GACTTTACGTTTAAGAAGACCCCAGTCAACAAACTGGGAATCCTATATTTAATTAATTATGATCAAATTTCATAGAAACTATCAGTGCATTGAA
AATCAAAGTGACTTGAACGACGGCGTTGATACTGCAAGGTCCTACATGCGGTCTGTGAAGACTACAAGAGGCTCATCGATCTGAAAAAAAAA
ACATCTGTGCAAATGTTTCTAGTTTGAATAAACTCAATCGGTTAAAC

Figure 4. (A) Schematic diagram of amplification of the 5´ and 3´-end from genomic DNA (GSP= gene specific primer). (**B)** Amplification of 5´ and 3´-end regions from cDNA. A 5´ UTR and two of 3´ UTR variants were found. The coding sequence of AAEL010824 gene (beginning and end) is shown in black; 5´ UTR is shown in red; the most common 3´ UTR is shown in blue; and the less common 3´UTR is shown in green.

Table 4. 3´ and 5´ RACE of AAEL010824 gene

AAEL010824 cloned no.	No. bases	Sequence
5´ RACE		**5´ UTR**
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	10	GTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	7	ATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	24	TTTTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	13	AGTGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	22	CTTGTGACATCCGTTATTCAAA**ATG**GAACTTCTACAGATATT.....
	17	GGAGTAGAAAATTCAAA**ATG**GAACTTCTACAGATATT.....
3´ RACE		**3´ UTR**
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	43	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCC
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	43	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCC
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	48	ATG…..…..**TAG** TTCATAGACAATGGCGTTTAAGCGAATAAAATATCGATTTCCCCTTGT
	726	ATG…..….. 97 bases splicing …..AGGGTAGT……..…… AAACTCAATCGGTTAAAC
	726	ATG…..….. 97 bases splicing …..AGGGTAGT……..…… AAACTCAATCGGTTAAAC

A synthesized cDNA of 5´end using oligo dT was used as a PCR template to confirm 5´end RACE results. PCR results showed that when using either 10824-ChR1 or 10824-ChR2 reverse primers with GeneRacer^{TM} 5´ primer, amplicons of expected 368 and 449 bp were detected. When the reverse primer 10824-ChR3 (115 bases away from 22 bases of 5´ends

sequence) or 10824-ChR4 (6 bases overlap within 22 bases 5´ends sequence) were used in PCR reactions, no amplicon was found. These PCR results indicated that 22 bp are 5´UTR upstream of the transcription start site (AUG) of AAEL010824 gene (Figure 3).

Development of an AAEL010824 promoter construct

After a length of 5´UTR of AAEL010824 was identified, 624 bp sequence of AEEL010824 was blasted against *Aedes* vectorbase genome. Although the first consensus TATA box was located at -993bp and CAT was nearby at -999 bp as shown in Figure 4, we went approximately 5 kb upstream of 5´UTR to using Promoter 2.0 Prediction Server to predict the promoter region of this gene.

In this experiment, iProof high-fidelity DNA polymerase from Bio-Rad® (Hercules, CA, USA) resulted in the best PCR amplification of this region. Sequencing results of the predicted promoter region were blasted against the *Aedes* genome. There were 7 different bases in 7 locations in amplicons of 2 different males as shown in table A1.

Overall for the pBac[3XP3-AAEL01824 EGFP-DsRedaf] promoter construct , I began reassembling 718 bp EGFP plus 266 bp SV40 poly A to pBac[3xP3-DsRedaf] in the unique cloning sites (*Asc*I/*Fse*I) plus 5 extra bases (5´-CCACC-3´) before the ATG start codon of EGFP. The pBac[3XP3-EGFP-DsRedaf] construct was then ligated with AAEL010824 promoter fragment at *Fse*I site. Eight out of 96 colonies (8.33%) contained our final constructs (Figure 6A). Finally, the plasmid construct was selected and sequenced to confirm promoter and reporter sequence regions including orientation. The construct sequence results were identical to the original sequence.

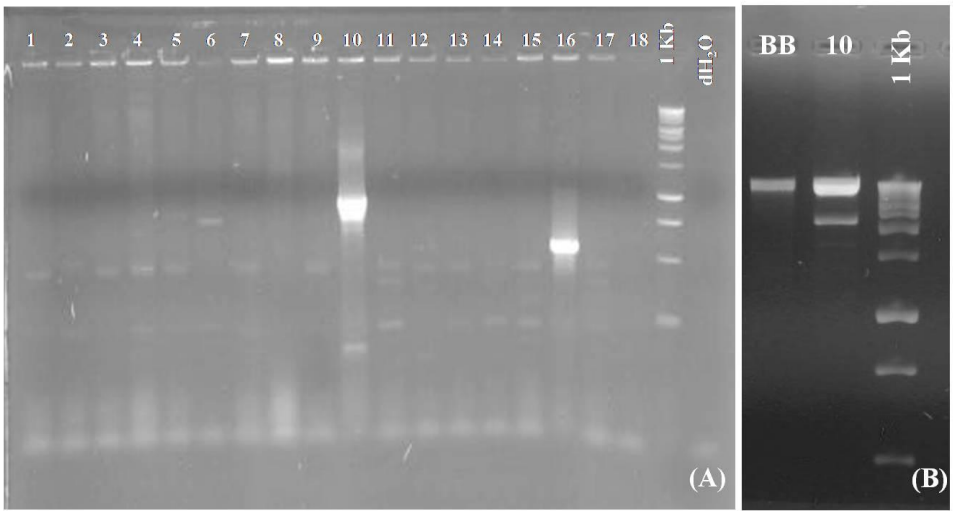


Figure 5. (**A**) Eighteen clones were screened for EGFP insertion in**)** pBac[3xP3-DsRedaf] (numbers indicate colony numbers). Colony number 10 was selected for confirming EGFP insertion by *Asc*I and *Fse*I digestion. **(B)** pBac[3xP3-EGFP-DsRedaf] clones were confirmed by digesting with *Sac*II and *Fse*I. Digested plasmid reactions were visualized on 1 % agarose gel electrophoresis. The correct clone of approximately 1,976 and 7,408 bp fragments were detected as shown in number 10. BB is pBac[3xP3-DsRedaf] plasmid without EGFP insertion.

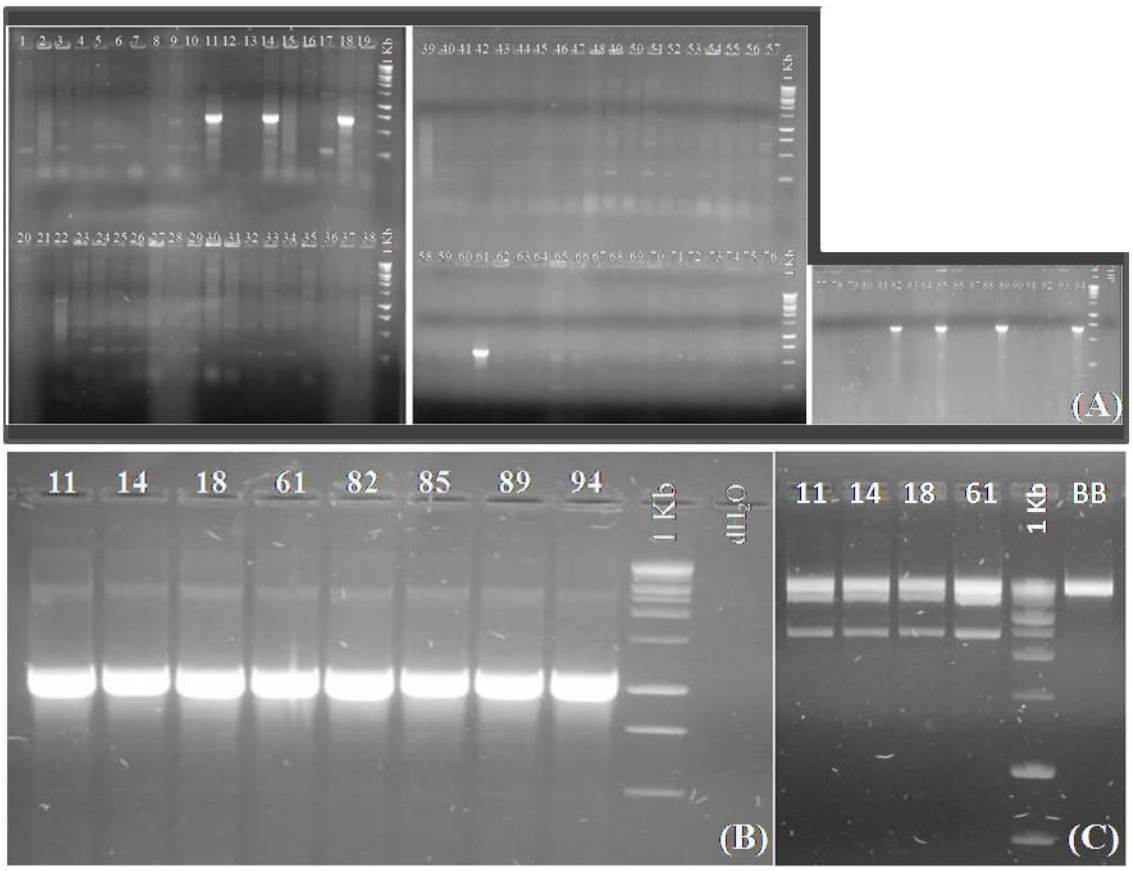


Figure 6. (A) 100 clones were screened for 5 kb predicted AAEL010824 promoter region in pBac[3xP3- EGFP-DsRedaf] plasmid. Correct clones were recognized by the amplification of a 756 bp. **(B)** Colonies number 11, 14, 18, 61, 82, 85, 89, and 94 were confirmed by using a second set of primers (DsRed-CF_1 and *Fse*I-EGFP-F_5) and correct clones were recognized by the amplification of an approximately 1 kb. (**C)** Confirmation of orientation of an insert from plasmids of 4 positive colonies were digested by *Sac*II and *Xho*I digestions and approximately 6.16, 4.02, and 2.27 kb fragments were visualized under 1% agarose gel electrophoresis. BB is pBac[3xP3-EGFP- DsRedaf] plasmid (backbone).

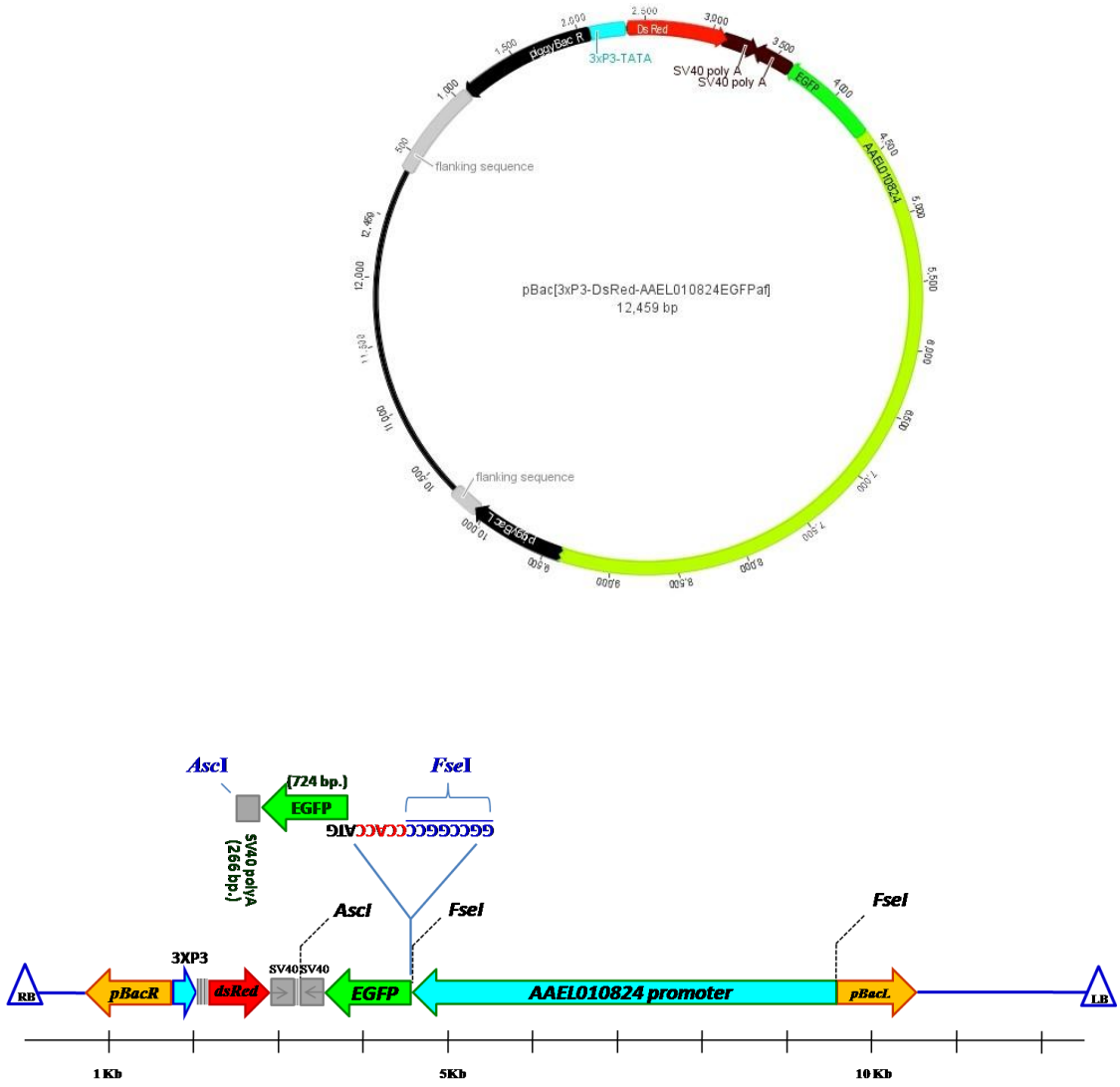


Figure 7. Overview of the final promoter construct: pBac[3xP3-AAEL010824EGFP-DsRedaf].

CONCLUSIONS

In this project, I first identified the entire coding sequence of AAEL010824 gene. There was some variation in the length of 5´ UTR, with the most common of 22 bases. In eukaryotes, an average length for 5´ UTRs in different taxa varies from 90-170 bases except for *Drosophila* in which the average is 288 bases (Rogozin et al. 2001). A previously reported database showed an unusually long 5'UTR (315 bp) of *Drosophila* (Pesole et al. 1996). From my results, the 5´UTR length of AAEL010824 was short when compared to the average lengths reported for other genes. However, a promoter study of a polyubiquitin gene in red flour beetle (*Tribolium castaneum*) reported that the length of 5´UTR in this gene was 68 bases (Lorenzen et al. 2002). Another possible explanation for this short 5´UTR is that the length of introns within 5´UTR is linked to regulation of gene expression. Genes expressed at lower levels normally have longer UTRs, whereas highly expressed genes usually have shorter UTRs as transcription of shorter non-coding regions costs less energy. The most highly expressed genes in both human and *Caenorhabditis elegans* (Castillo-Davis et al. 2002) have 5´UTR and many housekeeping genes that tend to be compact with fewer and shorter UTRs as well as shorter coding regions (Eisenberg and Levanon 2003, Vinogradov 2006). AAEL010824 is AG-specific gene in *Ae. aegypti* that has a high expression level (Sirot et al. 2011).

The goal of the second section of this work was to create an AG tissue specific construct. In addition to AAEL010824, three other AG -specific genes were studied (AAEL002000, AAEL002715, and AAEL009239); however, only AAEL010824 and AAEL002715 were found suitable for promoter constructs. AAEL002715 promoter development requires further study as 5´ UTR sequences in the predicted promoter region were slightly different at the 11 and 4

positions compared with vector base sequences in two individual males, respectively (data not shown). Sequences of this region may need to be confirmed with many *Ae. aegypti* male samples to determine the degree of variability. For AAEL002000, there were extra sequences of 494 bp in the middle of UTR predicted promoter region. Extra sequences were blasted against NCBI nucleotide BLASTs and results showed 100 % match to *Ae. aegypti* clone 321 "Pony Aa-A2 MITE repeat region", Genebank AF208865.1 (data not shown). Thus, AAEL002000 cannot be used for AGs-specific promoter construct. For AAEL009239, many attempts to obtain complete sequence of this gene were unsuccessful. DNA sequencing can fail in GC-rich regions; however the % GC content of this gene was only 31.48 %. This promoter may be re-examined in the future as a potential AGs-specific promoter.

Due to the problems with AAEL002000, AAEL009239, and AAEl002715 genes, I decided to go forward with the AAEL010824 promoter. Initially I attempted a three-way ligation with three DNA fragments; 5.031 Kb AAEL010824 promoter region (*Fse*I/*Sac*II), 1 Kb EGFP (*Sac*II/*Asc*I), and 6.345 Kb pBac[3xP3-DsRedaf] (*Fse*I/*Asc*I). This strategy was unsuccessful probably due to poor efficiency of ligation of these large DNA fragments. Moreover, during a process of screening transformation clones using colony PCR; there were many false positive results. This may have been due to remaining un-ligated DNA fragments from a ligation step retained on the plate. Unligated DNA contamination with this type of approach has been reported by others (Kitchin et al. 1990, Yang et al. 1998, Wybranietz and Lauer 1998, Agrawal and Roy 2008). A new strategy for AAEL010824 promoter construct was then created as described in the methods and the pBac[3xP3-AAEL010824EGFP-DsRedaf] construct was successfully obtained. This transgenic line with the AAEL010824 promoter has been confirmed to drive AGs expression.

CHAPTER 3

MODULATION OF SPERM AND SEMINAL FLUID QUANTITY BY MALE *AEDES AEGYPTI* IN RESPONSE TO FEMALE REPRODUCTIVE POTENTIAL

INTRODUCTION

In many animal mating systems, females are typically choosy and tend to mate with preferred partners. On the other hand, it is believed that males discriminate their choice of partners under certain conditions such as limited ejaculations, low fertilization rates or strong sperm competition (Dewsbury 1982, Johnstone et al. 1996, Kokko and Monaghan 2001, Nakatsuru and Kramer 1982, Parker 1982).

Theoretical models have predicted that sperm allocation patterns should be influenced by quality of potential females and that males should increase their sperm investment with increasing female fecundity (Galvani and Johnstone 1998, Reinhold et al. 2002). Gage and Barnard (1996) reported that cricket males (*Acheta domesticus)* transferred more sperm when encountering larger females. In spiny king crab (*Paralithodes brevipes*), ejaculate size of large males increased proportionally with increasing female size, but this trend was not observed with small males, indicating that small males could not increase ejaculate size by more than a certain level (Sato et al. 2006). Males of several insect species ejaculated volumes proportional to female body sizes (Johnson and Hubbell 1984, Gage and Barnard 1996, Wedell 1998, Parker et al. 1999). One study with the yellow dung fly showed that large males transferred more ejaculate to females during copulation resulting in greater fertilization success (Simmons et al. 1996), but smaller males compensated by spending more time in copulation with females (Simmons and Parker 1992, Parker and Simmons 1994).

In mosquito studies, larger *An. freeborni* males, collected from California rice fields, mated more often than smaller ones (Yuval et al. 1993). In *Ae. aegypti*, the number of total mature sperm in males was positively related to their body size and age (Ponlawat and Harrington 2007, 2009). In addition, older *Ae. aegypti* males under field conditions transferred more sperm to females than younger ones and the greater proportion of successful matings was positive correlated with female body size (Ponlawat and Harrington 2009).

In this study, I investigated whether *Ae. aegypti* males modulate the quantity of sperm and seminal fluid proteins they transfer to females in relation to female reproductive potential (as indicated by female size), the advantage of body size is also well known for female mosquitoes, as it is proportional to the number of gametes and fecundity as well as increased survival and blood-feeding success (Steinwascher 1982, Haramis 1985, Packer and Corbet, 1989, Washburn et al. 1989, Briegel 1990, Lounibos et al. 1990, Nasci 1990, Lyimo and Takken 1993, Renshaw 1994).

MATERIALS AND METHODS

Mosquito rearing. A Thai strain of *Ae. aegypti* originally established from mosquitoes collected from Soi Lat Krabang 36, Bangkok (15°7193′N, 101°752′ E), Thailand from May - June 2009 was used in this study. Mosquitoes were maintained in an environmental chamber set with a temperature range of 23.1 $\pm$ 2.7 ^{o}C, 79.2 $\pm$ 8.8% relative humidity (RH) with a 14-hours light (L): 10-hours dark (D) photoregime. Eggs of *Ae. aegypti* were hatched in a vacuum flask for 30 min with a small amount of diet (30 µg, 1:1 ratio of lactalbumin/brewer's yeast) added to the water. Larvae were held in the flask for 24 hrs at 28 ^{o}C until they were large enough to be sorted

into rearing trays. To obtain large, medium, and small body sizes, larvae were reared following the methods of Ponlawat and Harrington (2007). To obtain virgin mosquitoes, pupae were placed in individual tubes and separated by sex after eclosion. Mosquitoes were maintained in 5 L plastic cages with a 10% sucrose solution until experiments commenced. In each experiment, different cohorts of large and small virgin 3-5 day-old females and medium size virgin males were used.

Treatments 1-2: Male allocation of sperm and seminal fluid proteins to large and small females with no male competition. Three females of each body size were released into a 5 L bucket cage followed by one medium size male. Copulation initiation and duration were observed and recorded. Just prior to completion of copulation, the mating pair was removed with an aspirator and placed in a small glass tube (only mated females that experienced a full copulation of more than 5 sec were used in this experiment). The tube was labeled with female body size and mating number. A fresh virgin female of the same size and a virgin male were then added to the cage before the next mating. This method were repeated until 30 mated females had been obtained for each treatment (treatment 1= Large female No competition (LN), treatment 2=Small female No competition (SN)). A total of 30 samples of each size category were obtained. Tubes containing females for sperm quantitation were quickly placed on ice and then stored at -20^{o}C for dissection and staining. Tubes containing females for sfp quantification using western blot analysis of AAEL010824 (described below) were flash frozen on dry ice and stored at -80 ^{o}C.

Treatments 3-4: Male allocation of sperm and seminal fluid proteins to large and small females with male competition. At the same time, a second set of matings was undertaken using the same approach as above with the exception that clasperless males were added to the

cage as a proxy for male competition without the risk of fertilization. Claspers were cut off of a group of males so they could not grasp and mate with females following the methods of (Helinski and Harrington 2012). To aid visualization, the clasperless males were dusted with florescent orange dayglo power following the methods of Harrington et al. (2008). One clasperless orange dusted male was placed in the cage with females followed by the addition of one virgin male. Clasperless males were replaced if necessary with fresh clasperless males. A total of 30 samples of each treatment size category (treatment 3= large females with Competition (LC), treatment 4=small females with Competition (SC)) were obtained.

Treatment 5: Males held with male competition history since eclosion. To examine if males held with other males after eclosion affected their semen allocation patterns, we included one additional treatment in replicate 3 (ILC) where males were isolated after eclosion until the experiment commenced.

Control treatments. Additional controls buckets were set up with 24 virgin females (small and large held in size cohorts separately) with 8 clasperless males in each bucket. These were placed adjacent to the experimental cages as the mating experiment was conducted. Half of the large females from control bucket were removed after the experiment and flash frozen for use as virgin controls in the western blots. All small females and the other half of large females were dissected and inspected for sperm presence after brief anesthesia on ice to confirm that clasperless males could not mate.

Mating experiments were conducted over one day from approximately 08:00 to up to 18:00 with up to 4 observers conducting the matings at the same time. Three replicates were conducted. Female samples were analyzed for sperm numbers and sfp transfer as described below.

Sperm quantitation. In replicate 1 and 2, the spermathecae of females were dissected from thawed samples and gently teased apart with dissecting pins. Homogenization, allocation and staining of sperm was conducted from the published methods of Ponlawat and Harrington (2007) Briefly, spermathecae were placed into a multi-well slide containing 20 µl of PBS buffer. Each sample was torn open gently with pins followed by washing of pins with 20 µl twice of PBS to obtain the final stock volume of 60 µl. Sample solution was mixed and 4 µl of stock sample were spotted on multi-well slides (MP Biomedicals, LLC. OH, USA). Slides were air dried and fixed with 70% ethanol. After fixing, slides were stained with Giemsa dye (Sigma Chemical Co., MO, USA) for 1 hr then rinsed with distilled water and allowed to air dry. Mosquito sperm heads (stained pink by Giemsa) were counted using a phase contrast microscope. A total of 5 aliquots of 15 were counted and averaged, then multiplied by 15 to obtain the overall count. For replicate 3, the number of sperm in both the spermatheca and bursa were counted.

Sfp quantity transferred using AAEL010824. Female lower abdominal segments were homogenized in 18 µl of 2x sample buffer (20 ml of glycerol, 47.5 ml of H_2O, 20 ml of 20% SDS, 12.5 ml of 1M Tris pH6.8, 1 mg of bromophenol blue, and 10% of β–mercaptoethanol) and prepared for western blot. Fifteen µl of each homogenized sample was loaded into a 15% SDS polyacrylamide gel. Negative control virgin large females from the control treatments and positive control male samples (half accessory gland virgin males) were included. Western blots were performed following the procedure of Lung and Wolfner (1999) with slight modifications. A mixture of 5% dry milk in 1x TBST (1x TBS and 0.01% Tween-20) containing 1:2000 AAEL010824 polyclonal antibody (primary) or 1:5000 secondary antibody concentration were incubated at room temperature for 1 hr in each blocking solution. Tubulin antibody was used as an internal loading control at 1:60,000 of first and second antibody. Membranes were imaged

using Storm 860 Molecular Dynamics System (Amersham Biosciences SV Corp, CA, USA). The amount of AAEL010824 protein in each sample was estimated using Image Quant software of the Bio-RadGel Doc™ XR System (Bio-Rad, Hercules, CA, USA) as described in manufacturer's software protocol.

Data analysis. The entire experiment was replicated 3 times. In replicate 3, competition male cohorts were divided into two groups as described above: one where males were isolated after eclosion until the experiment commenced and one where males were group housed. All data were analyzed using SPSS v19 (IBM Armonk, NY). Before analysis, data were checked for normality. When possible, transformations were used to normalize the data. Data on copulation duration time were compared by treatments with Kruskal-Wallis ANOVA.

Body sizes were confirmed with wing length measurements of twenty samples of each male and female body size cohort (Briegel 1990, Nasci 1990) and analyzed with Kruskal-Wallis ANOVA. A Mann Whitney test was used to compare large and small body size females.

Univariate ANOVA with Tukey's HSD separation of means was employed for analysis of sperm and sfp transfer. Treatments were compared for each group between replicates, when no statistical differences were observed, and they were combined. Data on sperm quantity were compared by female body size and male competition status. Replicate 1 and 2 only included counts for the spermathecae, so they were analyzed separately from replicate 3 that included counts from both the bursa and the spermathecae.

Two outlying data points for sfp quantity (10824) were removed prior to analysis. Data were standardized by dividing by the intensity of the male 10824 control band within each gel. Data were then log-transformed and analyzed with Univariate ANOVA. The final models included gel, treatment, and all interactions including replicate when appropriate. Post hoc

separation of means was performed with Tukey's HSD test. In replicate 1, gel 3 was significantly different from the others and these data were removed from analysis. In replicate 3, significant variation by gel was observed even after normalizing each gel by male AG samples on that gel. Gel 2 and 3 were significantly different from the other 4 gels and were removed from the analysis. In replicate 3, for the comparison of males isolated after eclosion (ILC) and grouped males, a slight effect of gel was observed (p=0.05) with gel 4 significantly different from all others and gel 1 significantly different from all others. Given the marginal significance, I did not eliminate these gels from the analysis (Table A8-A9).

RESULTS

Mosquito body size

No significant differences within body size cohorts were found between the replicates for medium males (H=1.556, df=2, p=0.459), large females (H=0.541, df=2, p=0.763), and small females (H=4.203, df=2, p=0.122). Large females (wing length=2.94 $\pm$ 0.04sd mm) were significantly larger than small females (wing length=2.19 $\pm$ 0.10sd mm) for all experiments (U=0.0, p<0.001).

Copulation time

Copulation duration was uniform for different female body sizes and treatments (H=1.739, df=4, p=0.784; Figure 8). The average copulation time for males group housed after eclosion but presented with females individually (absence of male competition) was 10.68 $\pm$ 0.17 and 10.73 $\pm$ 0.23 sec $\pm$ se for large and small females, respectively. In males group housed and

presented with females in the presence of competition with other males, the average copulation time was 10.61 ± 0.19 and 10.76 ± 0.24 sec ± se for matings with large and small females, respectively. For the male group isolated after eclosion and presented to females with male competition, the average copulation time was 10.94 ± 0.37 sec ± se.

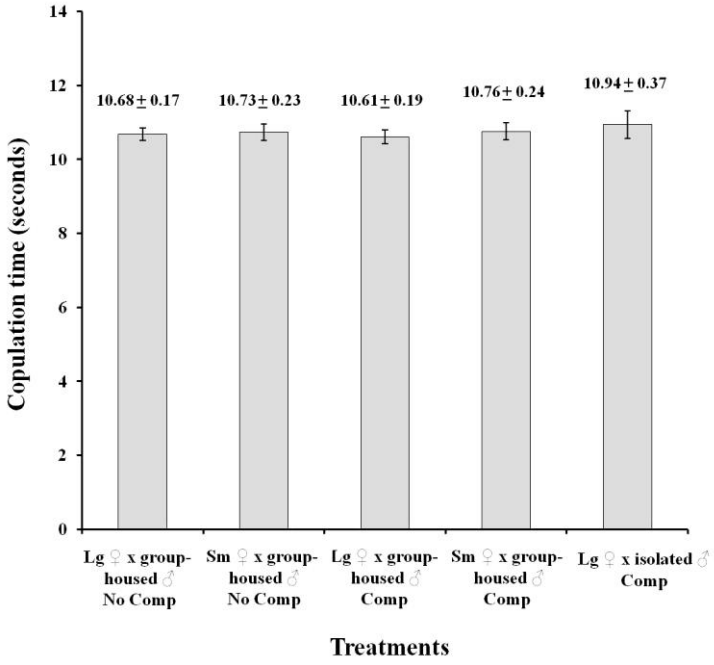


Figure 8. Copulation duration of five treatments.

Sperm transfer

No sperm was found in spermathecae females in all replicates of control treatments.

In replicate 1 and 2 where sperm in just the spermathecae was recorded, no significant difference in mean sperm counts between replicates was observed (ANOVA; F=1.27,df=1, p=0.263). However, I did find an effect of body size. The amount of sperm transferred was greater for large females versus small females regardless of whether males were in completion or not (ANOVA; F=21.57,df=3, $p < 0.001$;Tukey's HSD $p<0.001$; Fig 10). A table with detailed SPSS results is presented in the Appendix. When males were presented with only large size

females, no differences were detected in sperm numbers transferred for both male competition conditions as show in Figure 9.

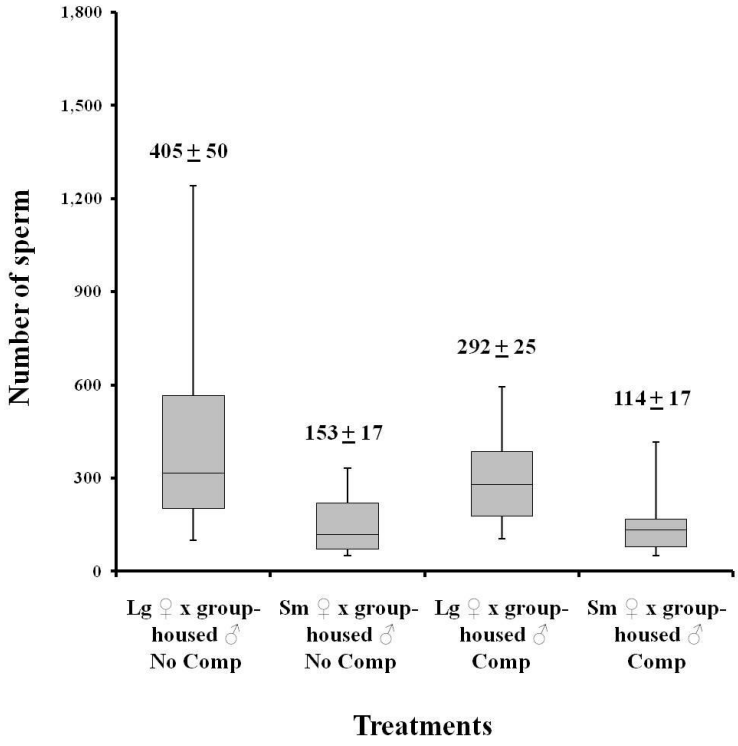


Figure 9. Number of sperm in spermathecae of females from four different treatments. Treatments: large, small females in the absence of male competition and large, small females in the presence of male competition.

For replicate 3, I realized that I needed to count sperm in both the spermathecae and bursa (counts were done for both rep 1 and 2 at the same time). Average numbers of sperm transferred to large and small females in the absence of male competition ranged from 1,635 to 3,950 and 1,023 to 3,025, respectively. No difference was found when male competition was present, the average number of sperm transferred ranged from 1,663 to 4,055 and 1,163 to 2,477 in large and small females, respectively. The range of sperm transferred by males that were kept isolated from other males since eclosion was similar as well (1,572 to 4,175).

The same trend was observed in replicate 3 no matter if males were in competition or not. Again, males transferred more sperm to larger females (ANOVA; F= 7.06, df=4, $p < 0.001$; Figure 10). No significant differences in sperm numbers were found for large females in the absence of competition ($\bar{x}$= 2,763.43 $\pm$ 206.84se), for males held grouped from eclosion in presence of competition ($\bar{x}$= 2,643.93 $\pm$ 182.38se), and for isolated males ($\bar{x}$= 2,783.33 $\pm$ 171.52se) (ANOVA; F (2, 42) = 0.174, p = 0.841; Figure 10). No difference was found for males that were isolated after eclosion and presented to females with male competition when compared to males that were not isolated (Tukey's HSD, p=0.076, SPSS results Table A5 in Appendix).

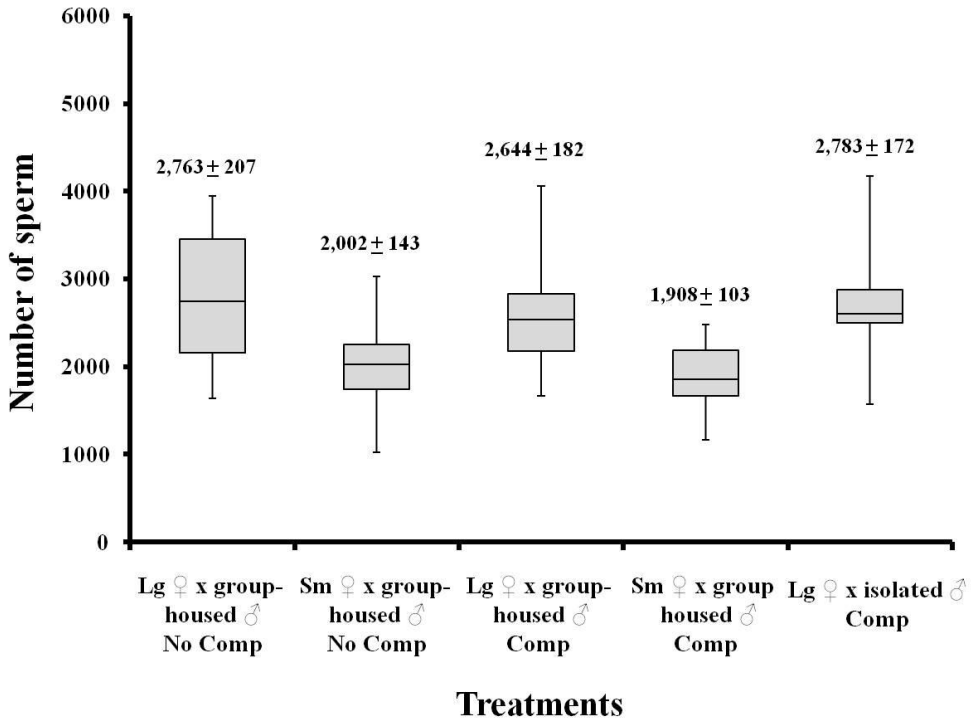


Figure 10. Number of sperm transferred to females in five different treatments. Treatments (group housed males): large, small females in the absence of male competition and large, small females in the presence of male competition. Treatment (male were isolated after eclosion until the experiment commenced): large females in the present of male competition.

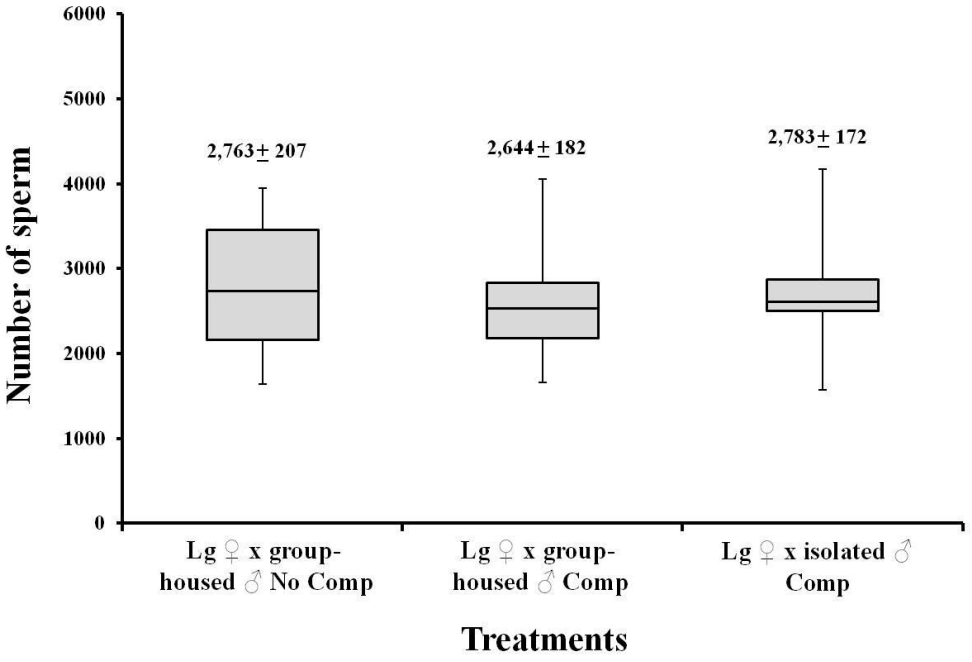


Figure 11. Number of sperm transferred into spermathecae and bursa of three different treatments. Treatment (group housed males): large females in the absence of male competition, large females in the present of male competition. Treatment (male were isolated after eclosion until the experiment commenced): large females in the present of male competition.

Seminal fluid proteins (AAEL010824) transfer

In replicate 1 and 2, no significant difference in AAEL010824 (intensity) in mated females was observed among treatments (F=1.43, df=3, *p*=0.24; SPSS results in Appendix Table A6; Figure 12). Western blot results are presented in Figure 13.

No significant difference by female body size was observed for replicate 3A (F=2.1, df=3, *p*=0.122; SPSS results in Appendix Table A7; Figure 14). Replicate 3 western blot results are shown in Appendix Figure A2-A3.

For the comparison of males isolated after eclosion vs. grouped males (replicate 3B), no significant differences by female body size were found (F=1.667, df=2, *p*=0.203; SPSS and western blot results in Appendix Table A8-A9; Figure 15).

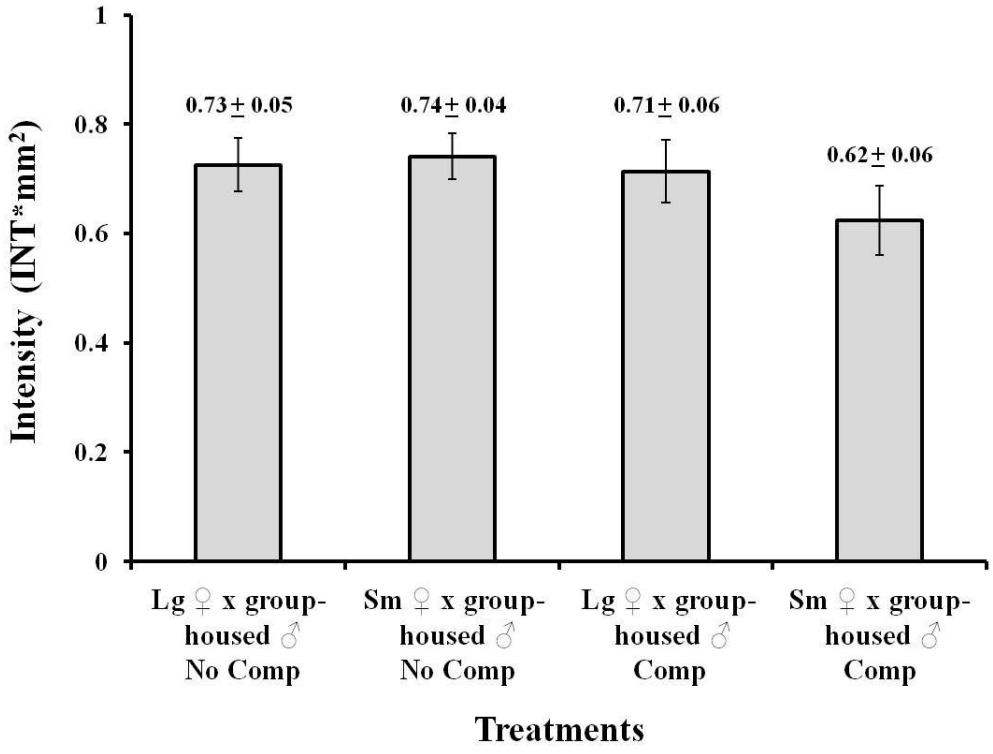


Figure 12. The amount of sfp (AAEL010824) transferred into females indicated by AAEL010824 intensity and normalized with intensity of male 10824 controls in four different treatments of replicate 1 and 2. Large and small females in the absence of male competition, and large and small females in the present of male competition.

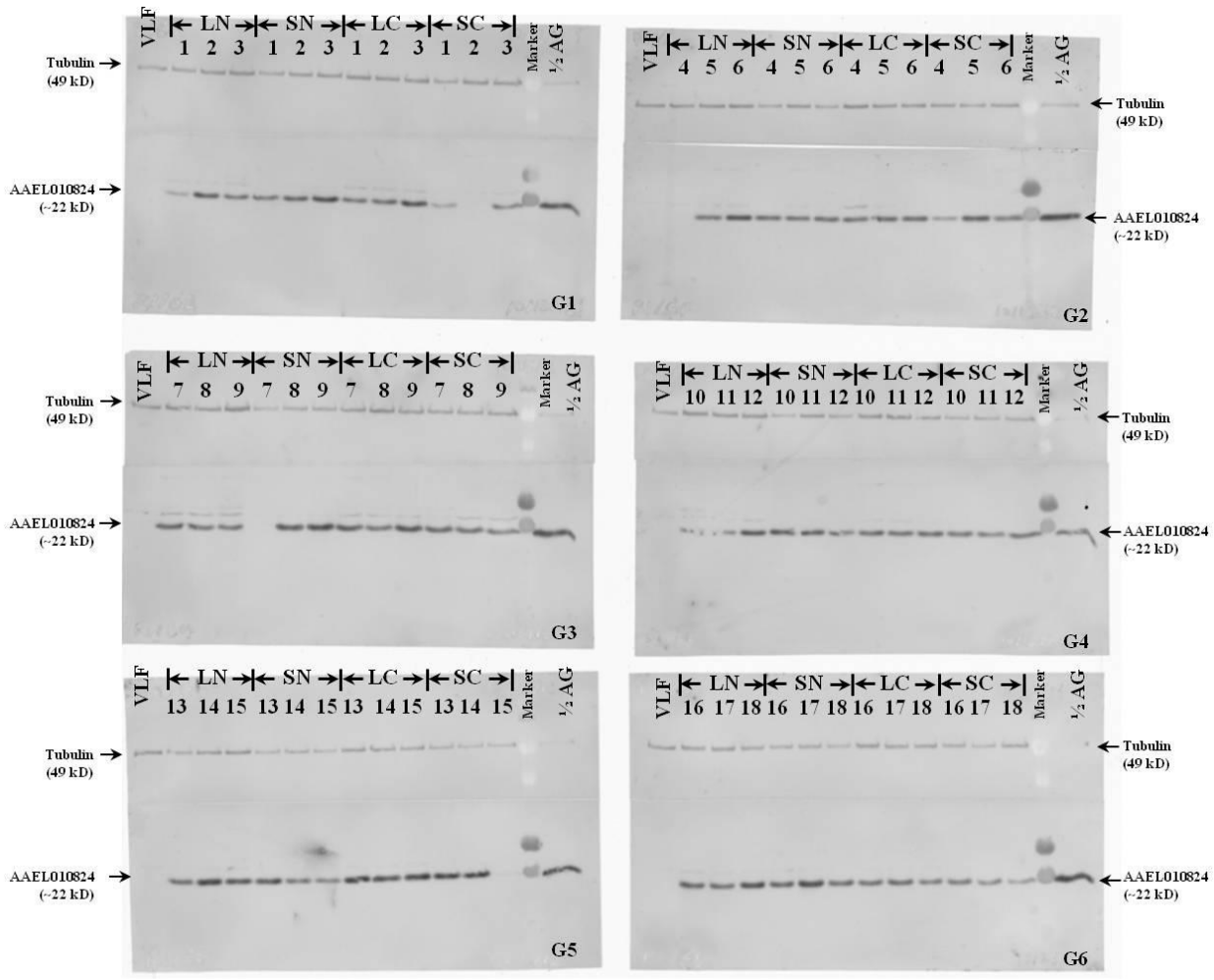


Figure 13. Western blot results of replicate 1. Treatment: large (LN), small (SN) females in the absence of male competition and large (LC), small (SC) females in the presence of male competition (G1-G6 = gel 1-6); tubulin (49 kD) and AAEL010824 (~22kD), Precision Plus Protein™ Dual Xtra Standards marker (Bio-Rad). Lane 1-18 = lower abdominal segments of one female from each treatment, VLF = lower abdominal segments of a virgin large female from the control treatment, and ½ AG = A half accessory gland of virgin males.

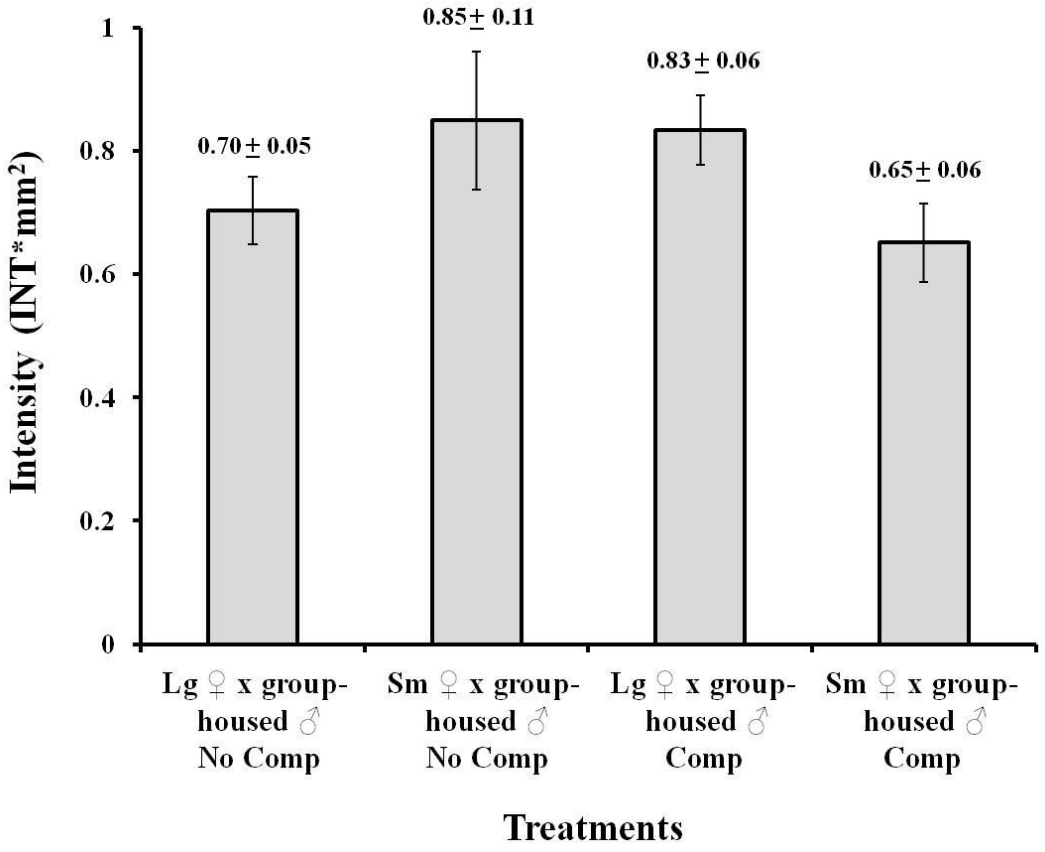


Figure 14. The amount of sfp (AAEL010824) transferred to females as indicated by measuring intensity of AAEL010824 and normalizing with intensity of male 10824 control bands in four different treatments of replicate 3A. Large, small females in the absence of male competition, and large, small females in the present of male competition.

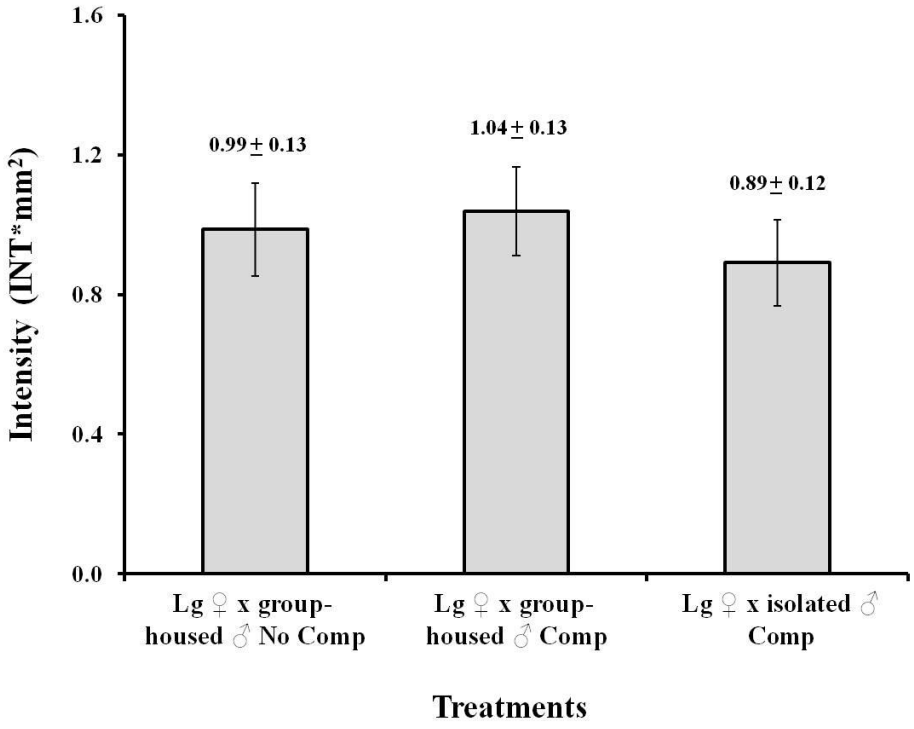


Figure 15. The amount of sfp (AAEL010824) transferred to females as indicated by measuring intensity of AAEL010824 and normalizing with intensity of male 10824 control bands in three different treatments of replicate 3B. Treatment (group housed males): large females in the absence of male competition, large females in the presence of male competition. Treatment (males were isolated after eclosion until the experiment commenced): large females in the presence of male competition.

CONCLUSIONS

My results indicate that male *Ae. aegypti* transferred more sperm to large females than to small ones as was demonstrated previously by Ponlawat and Harrington (2009). A body size is an indicator of female reproductive fitness. These results suggest that males have the ability to adjust the amount of gametes they invest in females based on reproductive potential. This result is interesting given that the amount of sperm males transferred was more than enough for large females. Sperm numbers transferred even to small females in my study would have been adequate for large females to utilize for lifetime egg production. Allocation of greater number of male gametes to large females has been described previously for many insect species and other animals (Wedell and Cook 1999, Cordero 2000, Hunter et al. 2000, Pizzari et al. 2003, Hunt et al. 2004, Pound and Gage 2004, Perez-Staples and Aluja 2006, Rubolini et al. 2006, Sato and Goshima 2007, Cornwallis and O'Cornor 2009).

Sperm counts were much lower than expected in Replicates 1-2 because I only counted sperm in the spermathecae and not enough time had probably elapsed from mating to flash freezing in order for sperm to reach the spermathecae. Despite these lower numbers, my results from replicates 1-2 followed a similar trend to replicate 3 where I counted sperm in both the bursae and spermathecae. Sperm counts from replicate 3 were similar to those reported previously by Ponlawat and Harrington 2009.

I did not find any effect of male competition on sperm allocation in this study even when comparing between males isolated after eclosion with those group housed males with other males. In contrast, many studies in insects have found that male ejaculate size increased relative to the risk of male-male competition (Gage 1991, Gage and Barnard 1996, Marconato and Shapiro 1996, Nicholls et al. 2001, Schaus and Sakaluk 2001).

The lack of differences by male competition may have been due to the way competition was presented in my study. I used clasperless males, which may not be as realistic as viable males. However, including more than one male in the experiment would not have been feasible, since matings had to be observed and it would have been difficult to keep track of more than one male. In addition, I only presented one clasperless male in the cages. If I had used more males, I may have seen an effect. During a pilot experiment, I did try to release three clasperless males into the mating cage with a normal male, but mating was challenging to observe. The sex ratio (male: female) in my experiments was probably higher for the female ratio that in nature. I used 1male: 6 females and 1:3 in none and male-competitive conditions, respectively. In nature, sex ratios are closer to 1:1 and the average numbers of males found resting inside houses can vary considerably ranging from 0 to 20 depending on the house and region of the world (Harrington et al. unpublished data from Thailand, Mexico, and Puerto Rico, Scott et al. 2000, Garcia-Rejon et al. 2008). Future studies should explore more realistic sex ratios, densities and should be conducted under field conditions if possible.

No significant difference was found between female size and competition in the amount of AAEL010824 (sfp) transferred into females. However, this could have been due to limitations in the sensitivity of the western blot or the intensity software. An ELISA, if available, would have been more sensitive. Sirot et al. (2009) reported a sensitive ELISA-based method for quantifying sfps level in *Drosophila melanogaster*. In addition, AAEL010824 may not be the best candidate for sfp quantitation and future studies may want to explore the use of several other sfps.

I found no significant relationship between copulation duration and sperm or AAEL010824 transferred in *Ae. aegypti*. Similar results for this species have been reported

(Helinski and Harrington 2011). In addition this trend was described for *Anastrepha obliqua* (Diptera: Tephritidae) (Perez-Staples and Aluja 2006) and other insects (Qazi et al. 1996, Taylor et al. 2000, Fritz 2004, Garcia-Gonzales and Gomendio 2004, Snow and Andrade 2004, Harmer et al. 2006). These results suggests that time spent during copulation may have other functions aside from sperm transfer.

In conclusion, these experiments provide clear evidence that male *Ae. aegypti* males transfer more sperm to large females. However, I found no evidence for differences in sperm or sfp allocation by males under the different competition conditions used in my study.

CHAPTER 4

AEDES AEGYPTI MALE MATING PREFERENCE FOR FEMALES OF VARYING FITNESS STATUS IN SEMI-FIELD CAGES IN THAILAND

INTRODUCTION

Body size is a basic factor influencing mating success for both sexes in many insects including mosquitoes, *Ae. aegypti*, *An. gambiae* (Okanda et al. 2002, Ponlawat and Harrington 2009). A wide range of adult *Ae. aegypti* body sizes are found in nature (Schneider et al. 2004) and may be important in mating preference or dynamics. Wing length is usually used as an indicator of adult mosquito body size in mosquitoes (Nasci 1990) and the body size has been reported to influence fecundity and other bionomic factors in mosquito populations (Haramis 1983, Nasci 1987). In females of several mosquito species, those with large body size had higher blood-feeding success and greater longevity survival rate than small females (Nasci 1990). Briegel (1990) found that blood meal size and fecundity were positively correlated with body size in *An. albimanus* Wiedemann, *An. gambiae* s.1, and *An. stephensi.* Similarly, Packer and Corbet (1989) showed that large *Ae. punctor* females had greater longevity than small females. A positive relationship between body size and fecundity was reported in females of *Ae. aegypti* (L.), *Ae. sierrensis* (Ludlow), and *An. gambiae* (Giles) *sensu lato* populations (Steinwascher, 1982;Washburn et al., 1989; and Lyimo and Takken 1993). Bock and Milby (1981), Reisen (1975), Renshaw et al. (1994), Bradshaw and Holzapfel (1992,1996) reported that large *Culex tarsalis* Coquillett, *An. stephensi* Liston, *Ae. cantans*, and *Ae. aegypti* females produced a greater

number of eggs. In addition larger mosquitoes are more efficient fliers than smaller individuals (Nayar, 1969).

Assortative mating is a nonrandom mating pattern where there is a tendency for phenotypically similar individuals to mate with one another more frequently than would be expected under a random mating pattern (MacDougall and Montgomerie 2003). One of the most commonly observed assortative mating patterns in animals is by body size (Crespi 1989).

Assortative mating has rarely been studied in mosquitoes (Manning 1975, Gwynne 1981, 1984, Marshall 1982, Rutowski 1982, Hieber and Cohen 1983, Ridley and Thompson 1985, McLain and Boromisa 1987). In this study, I investigated male mating preferences for female body size in the field in Thailand. I tested the hypothesis that assortative mating takes place in *Ae. aegypti*, such that both sexes preferentially mate with the largest mates available.

Three experiments were conducted. In the first experiment, female mating behavior and assortative mating by male body size was observed on a small scale. The results from this first experiment were confirmed by the second and third experiments on a bigger scale. All experiments were conducted under semi-field conditions using an F_1 generation of mosquitoes.

MATERIALS AND METHODS

Mosquitoes and rearing procedure

Ae. aegypti used in this study were the F_1 generation from 4^{th} instar larvae and pupae collected from containers in Samutprakarn, Thailand (13° 36.520´N, 100° 37.240´E; Figure 16). Collected larvae and pupae were maintained at the field site under ambient conditions prior to introduction into the field cage. F_1 eggs were collected and used for the experiments. Adult mosquitoes were continuously supplied with a 20% sucrose solution.

Figure 16. Larval collection sites in Samutprakarn, Thailand (13° 36.520´N, 100° 37.240´E).

Unlabeled mosquito rearing Eggs of *Ae. aegypti* F_1 generation were hatched in a vacuum flask for 30 min. Thirty milligrams of *Aedes* food (1:1 ratio of lactalbumin/brewers yeast) was added to the flask and held overnight at room temperature. Mosquitoes were reared to achieve large (male=2.169 $\pm$ 0.061sd mm, female=2.692 $\pm$ 0.085sd mm) or small body sizes (male=1.724 $\pm$ 0.096sd mm, female=2.105 $\pm$ 0.086sd mm) mosquitoes by changing density and diet. To obtain large body size mosquitoes, 100 of L_1 stage larvae were placed into a 22 x 34x10-cm plastic tray (Thanasupe® no. T4-72, Thailand) containing 1 liter of distilled water and *Aedes* diet (38, 75, 113, and 150 mg) was added into trays on day 1, 3, 4, and 5, respectively. To yield

small adults, 400 L_1 stage larvae were placed into each tray and 20, 37.5, 56.5, 75, and 56.5 mg of *Aedes* food were added on day 1, 3, 4, 5 and 6 respectively.

Stable isotope labeling ^{15}N-glycine (NLM-202-1, Cambridge Isotope Laboratories Inc, Andover, MA, USA) or ^{13}C-glucose (CLM-1396-1) was used to label adult male mosquitoes as described by Helinski et al. (2008, 2012). On day one of larva rearing, 41.1 mg of ^{15}N-glycine (i.e., 15% enrichment based on nitrogen content in diet) were added into larval water. For ^{13}C-labelling, to avoid the bacterial growth in the water tray, a stock solution of 144.6 mg ^{13}C-glucose in 50 ml water was made (i.e., 25% enrichment based on carbon content in diet) and added into rearing water at 30 and 20 ml at day 1 and 3, respectively. Additionally, for the control treatment, a tray per each body size cohort was prepared with non-labeled males. Pupae were collected daily and separated into single tubes to obtain virgin males. After adult males emerged, 5L cages were used to maintain virgin males until the start of the experiment.

Experimental design

Three experiments were conducted in a house at Samutprakarn, Thailand (13° 36.520´N, 100° 37.240´E) during July to mid August. The first experiment was set up in 12 L plastic mating cages (Picnic® 321-2, Thailand), while the second and third experiments were performed in a large screened bed-net cage (170 × 185 × 170 cm: 5.36 m^3) with a white sheet on the floor (Figure 17). Three edges of the bed-net were sealed by taping it to the floor and one open edge was used as an entrance. Hobo data loggers (Onset, Bourne MA, USA) were used to record hourly temperature and humidity inside the cages. In all experiments, the mosquitoes released were virgin and between 3-5 days of age.

Figure 17. Large screen bed-net cage (170 × 185 × 170 cm: 5.36 m^3) with the white sheet on the floor was used in the second and third experiments.

Experiment 1: Female mating behavior and *Ae. aegypti* assortative mating.

Experiment 1.1 Large males with large and small females.

Two large unlabeled males were released into a 12 L plastic mating cage (Picnic® no.321-2, Thailand). One large and one small female were released into the mating cage. Female mating behavior such as kicking, twisting her abdomen away from the male, and evasive flight were closely observed and when the copulation occurred, a mouth aspirator was used to transfer the pair into a tube. The tube was put on ice for later body size confirmation. All remaining mosquitoes in the cage were removed and discarded. A new set of mosquitoes was then added into mating cage. The experiment continued until 50 mating pairs were observed. Three days of mating were set up and approximately 150 couples were obtained in this experiment.

Experiment 1.2 Small males with large and small females.

Experiment 1.2 was conducted as described above in experiment 1.1 except two small males were used instead of large males.

Experiment 1.3 Large and small males with large and small females.

In this experiment, instead of releasing the same size of males into the mating cage, one large and one small male were released into the cage. The same mating conditions as described above for experiment 1.1 were followed.

Experiment 2: Timing of assortative mating dynamics for large and small *Ae. aegypti* males.

Experiment 2.1 Timing of assortative mating dynamics for large *Ae. aegypti* males.

Three bed net cages were set up with 10 large unlabeled males released inside with 10 unlabeled of large females and 10 small females. The sex ratio between males and females in this experiment was 1:2. After releasing mosquitoes for 30 min (cage 1), 60 min (cage 2), or 90 min (cage 3), mosquitoes were collected using a backpack aspirator and all females were anesthetized on wet ice. The spermathecae were dissected and examined under a compound microscope for evidence of insemination (Figure 18). A right wing was taken from each female to determine size. Data were analyzed to determine if large males preferentially mate with large females. Three replicates were conducted.

Experiment 2.2 Timing of assortative mating dynamics in small *Ae. aegypti* males.

This experiment was set up as described for Experiment 2.1 with small males instead.

Figure 18. Spermathecae were dissected using small forceps and insect pins (0.35 mm diameter) under dissecting microscope.

Experiment 3: Assortative mating using stable isotopes.

Five ^{15}N-labeled large virgin males and 5 ^{13}C-labeled small males were released into a bed net cage containing 5 unlabeled large and 5 unlabeled small virgin females. After 24 hours, all mosquitoes were collected and anesthetized on ice. Insemination was confirmed as described previously. Mated female samples were collected and processed following the methods of Helinski et al. (2012). Briefly, spermathecae from individual females were transferred to a small piece of quartz paper using a fine brush and placed in a tin cup. Tools were cleaned twice with

ethanol after every dissection to prevent contamination. A spike solution (i.e., consisting of sucrose [for C] and ammonium sulphate [for N]) was added to each sample to attain sufficient nitrogen and carbon to be above the detection limit of the isotope ratio for mass spectrometry. Three replicates were performed with switching of ^{15}N-labeled, ^{13}C-labeled and unlabeled males by body size. A control group consisted of 20 males of each labeled with unlabeled 10 large and 10 small females in 5 L cages. Samples were submitted to the UC Davis stable isotope facility (Davis, CA, USA) for analysis.

Statistical analysis All data were analyzed using SPSS v19 (IBM Armonk, NY). Body sizes were confirmed with wing length measurements of thirty sub-samples of each male and female body size cohort (Briegel 1990, Nasci 1990). Data were analyzed with Kruskal-Wallis ANOVA. A Mann Whitney test was used to compare large and small body size females and males. Data of the number of large or small males that copulated with either large or small females in all replicates from experiment 1.1 and 1.2 were analyzed using χ^2 goodness-of-fit test. In experiment 1.3, χ^2 was used to compare each set of body size (large versus small) males that copulated with (large versus small) females. A Bonferroni's correction was applied to six sets of body size mating data in which each *p*-value was multiplied by six. In experiment 2, the number of inseminated females was analyzed using χ^2 test. In experiment 3, isotope results were compared using generalized estimating equations (GEE) as described by Helinski et al. (2012).

RESULTS

Mosquito body size

No significant differences were found in wing length of either males or females within a body size class between the different replicates with my larval rearing methods: Large males (H=0.379, df=2, p=0.827), small males (H=1.096, df=2, p=0.578), large females (H=2.287, df=2, p=0.319), and small females (H=2.813, df=2, p=0.245). Large males (2.169 $\pm$ 0.061sd mm) were significantly larger than small males (1.724 $\pm$ 0.096sd mm) for all experiments (U=0.0, p<0.001). Large females (2.692 $\pm$ 0.085sd mm) were significantly larger than small females (2.105 $\pm$ 0.086sd mm) for all experiments (U=0.0, p<0.001).

Experiment 1: Female mating behavior and *Ae. aegypti* assortative mating.

A total of 150 matings were obtained and the majority of large males (116 out of 150) copulated with large females (χ^2 (1, N=156) = 37.026, p<0.001; Figure 19A). In the case of small males, no significant difference was found between mating with large (73) and small females (82), respectively χ^2 = 0.523, p=0.470; Figure 19B).

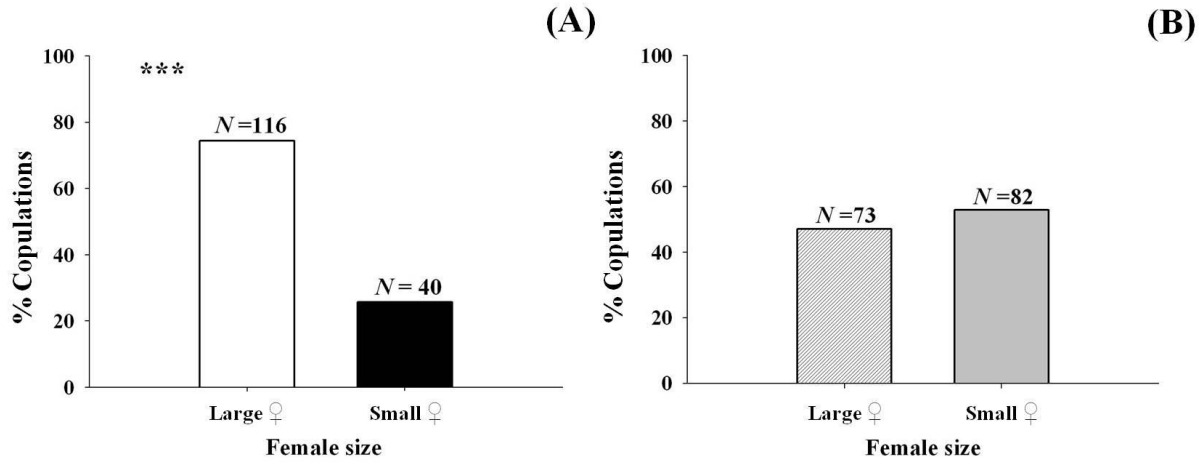


Figure 19. Assortative mating of **(A)** large *Ae. aegypti* males with large and small females in Thailand (χ^2 (1, *N*=156) = 37.026, *p*<0.001) **(B)** small *Ae. aegypti* males (χ^2 (1, *N*=155) = 0.523, *p* = 0.470). Significant differences are indicated by ***.

When one of each male size cohort were released simultaneously into a mating cage, differences in copulation with females by body size was observed with four different groups of matings (large females mated with large males (LFxLM), large females mated with small males (LFxSM), small females mated with large males (SFxLM), and small females mated with small males (SFxSM)); χ^2 (3, *N*=154) = 40.442, *p*<0.001 (Figure 20A). Large and small females had equal chances to mate with males, χ^2 (1, *N*=154) = 0.104, *p*=0.747 (Figure 20B). When large females were held alone, they preferred to mate with large (N=67) rather than small (12) males, χ^2 (1, *N*=79) =38.291, *p*<0.001 (Figure 20C). Small females examined alone were equally likely to mate with either large (33) or small (42) males, χ^2 (1, *N*=75) = 1.080, *p*=0.299 (Figure 20D).

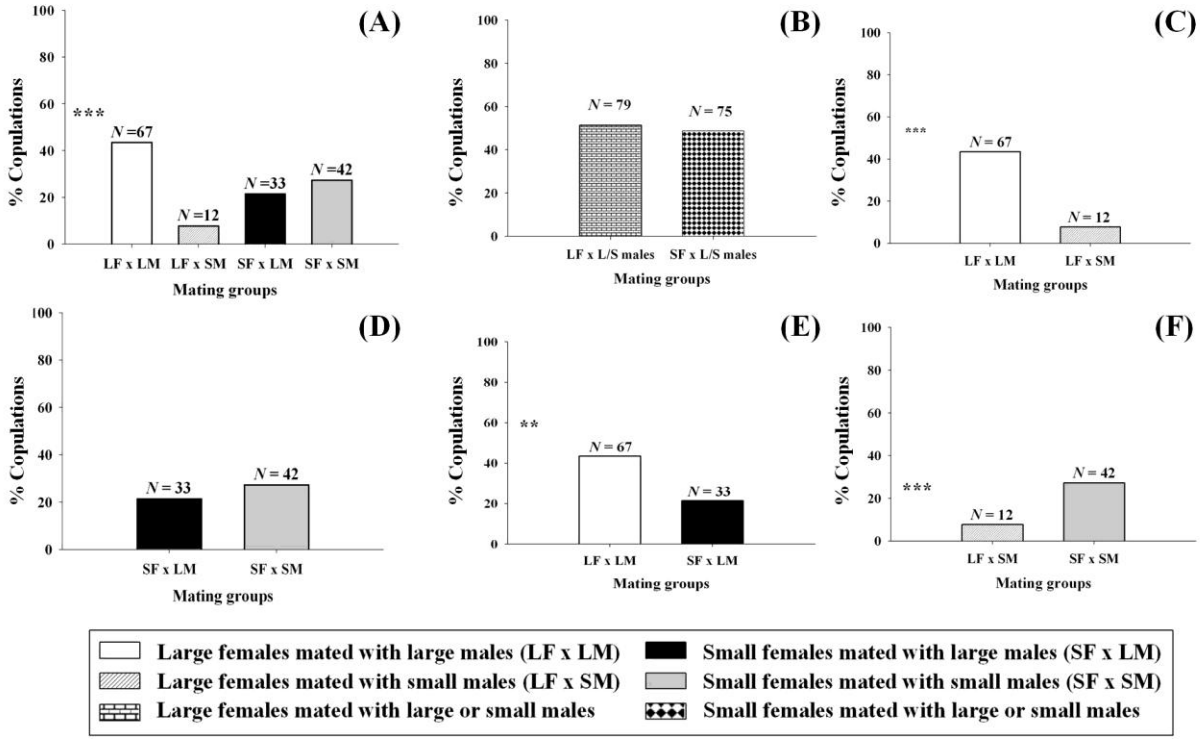


Figure 20. Assortative mating of large and small *Ae. aegypti* males with large and small females in Thailand. **A)** Overall chance of large or small males mated with large or small females; χ^2 (3, N=154) = 40.442, p<0.001. **B)** The mating preference of large or small females; χ^2 (1, N=154) = 0.104, p=0.747. **C)** The mating preference of large females; χ^2 (1, N=79) =38.291, p<0.001. **D)** The mating preference of small females; χ^2 (1, N=75) = 1.080, p=0.299. **E)** The mating preference of large males; χ^2 (1, N= 100) =11.560, p=0.001. **F)** The mating preference of small males; χ^2 (1, N = 54) = 16.667, p<0.001.

Large males when examined alone preferred to mate with large (67) females rather than with small (33) ones, χ^2 (1, N=100) =11.560, p=0.001 (Figure 20E). When examined alone, small males copulated significantly more with small rather than large females, χ^2 (1, N = 54) = 16.667, p<0.001 (Figure 20F). Overall comparison of the four different mating cases suggested that large males preferred to mate with large females (67) even though small males and females were present (Figure 20A; p<0.001), and large females did not prefer to mate with small (12) males when large males were nearby (Figure 20A, 21F; p<0.001, p<0.001).

Experiment 2: Timing of assortative mating dynamics in large and small *Ae. aegypti* males.

Experiment 2.1 Timing of assortative mating dynamics in large *Ae. aegypti* males**.**

Over the shortest time interval (30 min), large males inseminated large (15) females more often than small (4) females, χ^2 (1, N=19) = 6.368, p=0.012 (Figure 21A). When the time period was longer (> 30 min), there was no significant difference of large males inseminated with both female sizes; 60, χ^2 (1, N=37) = 3.270, p=0.071 and 90, χ^2 (1, N=40) = 2.5, p=0.114 minutes (Figure 21A).

Experiment 2.2: Timing of assortative mating dynamics in small *Ae. aegypti* males.

No significant differences were found for female body size at all time points when small males were examined alone; 30, (χ^2 (1, N=20) = 0.8, p=0.371), 60 min, (χ^2 (1, N=35) = 0.257, p=0.612), and 90 min, χ^2 (1, N=38) = 1.684, p=0.194) minutes (Figure 21B).

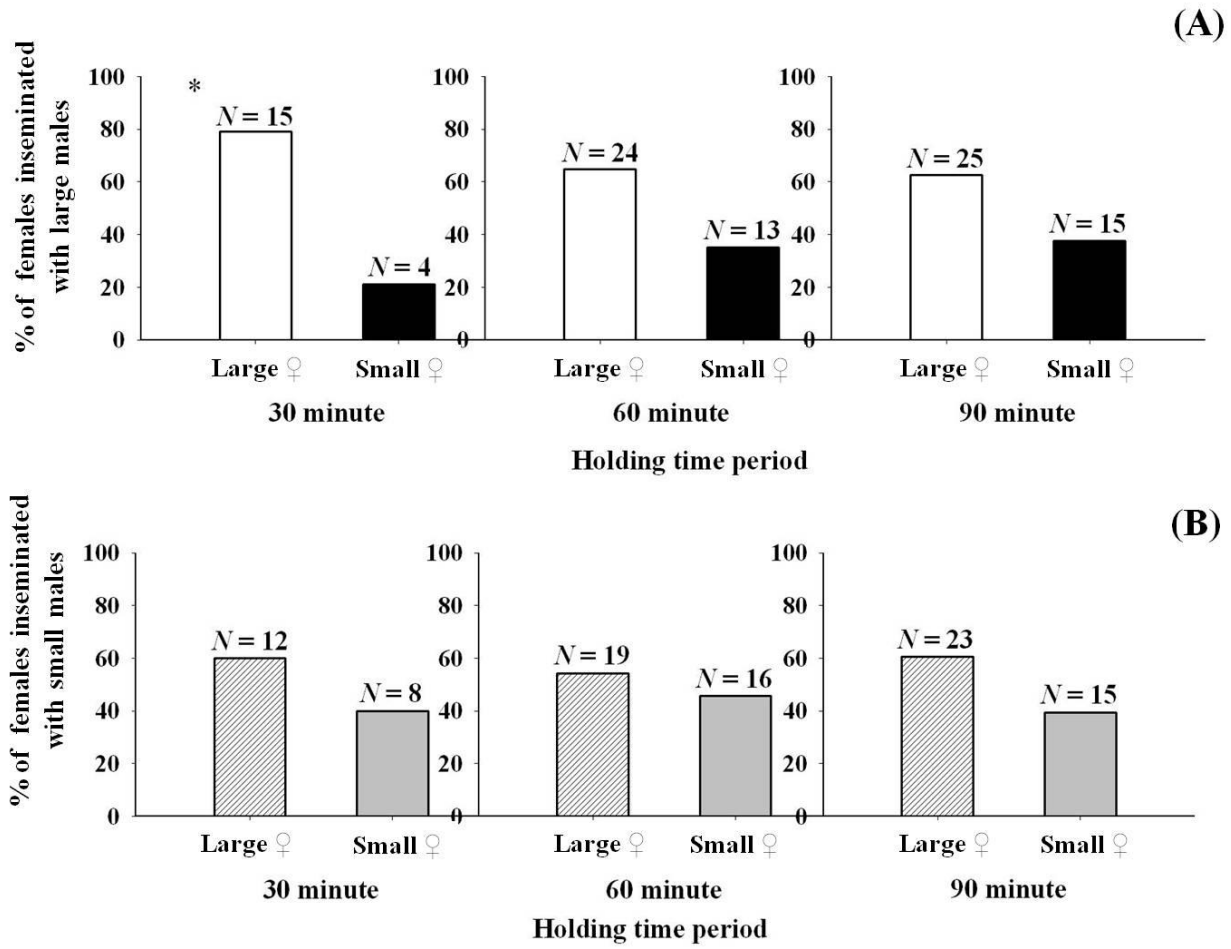


Figure 21.Assortative mating of large **(A)** and small **(B)** *Ae. aegypti* males with large and small females in indoor bed-net cages in Thailand. Ten large or small virgin males were released in the net containing 10 virgin large and 10 small females. Mosquitoes were removed after 30 (cage 1), 60 (cage 2), and 90 (cage 3) mins (*$p<0.05$).

Experiment 3: Assortative mating using stable isotopes.

All isotope analysis results are provided in detail in Appendix 1. Results from ^{13}C-labeled (^{13}C-LM) and 5 ^{15}N-labeled (^{15}N-SM) male experiments demonstrated that females mated with large males more frequently than small males (p<0.001). The estimated marginal means of 95% Wald Confidence Interval (95% WCI) of large females ($\bar{x}$ = 0.8) suggested that large females preferred to mate with large males and small females mated equally with males of both sizes ($\bar{x}$ = 0.53) (Figure 22A). When the order of isotope labeling was switched to ^{15}N –labeled large males (^{15}N-LM) and ^{13}C-labeled small males (^{13}C-SM), no differences were found, females did not mate more frequently with large males (p=0.222) (Figure 22B).

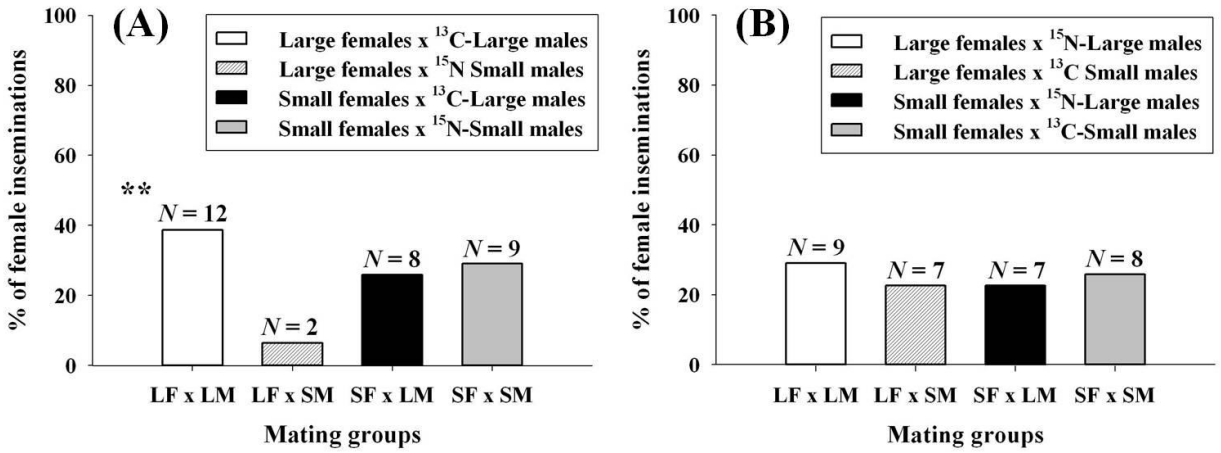


Figure 22. Assortative mating of **(A)** ^{13}C-labeled large and ^{15}N-labeled small *Ae. aegypti* males; p<0.001. **(B)** ^{15}N-labeled large and ^{13}C-labeled small *Ae. aegypti* males mated with large and small females in Thailand; p=0.222 (**p<0.001 in GEE).

CONCLUSIONS

These experiments provide some evidence that assortative mating by size may take place in *Ae. aegypti*. The experiments in this study were performed in Samutprakarn, Thailand during end of July to mid August 2011 using F_1 generation of mosquitoes derived from field-collected larvae and pupae. In addition my experiments were conducted in a human house (close to the field-collected areas) that represented the actual habitat of this species. Although these field cage results may not be typical of free-ranging mosquitoes, studying assortative mating in free ranging populations is not technically possible. Specifically, this is the first study to attempt to investigate mating preference of *Ae. aegypti* in Thailand.

Female body size is important for female mating success and male body size is positively correlated with male sperm capacity (Ponlawat and Harrington 2007). The results from experiment 1 indicated that large males copulated more frequently with large females, due to the higher proportion of large males mated with large females (116 out of 150 mating pairs; 77.33%). When small males were substituted instead of large males, I did not find any differences in mating patterns; the proportions were uniform for large (73 out of 150; 48.67%) or small females (82 out of 150; 54.67%), suggesting that small males may not have a mating preference.

While assortative mating has been reported in natural animal populations, its causes are rarely known (Crespi 1989, Foote and Larkin 1988, Brown 1990, Robertson 1990). Often, it is not clear if assortative mating occurs because of male or female preferences, and it may depend on both sexes. Darwin (1871) and Ridley (1983) explained hypotheses of assortative mating by mate choice as a result of males, females, or both sexes choosing large mates because they benefit reproductively and are differently capable of exercising choice. My results in experiment

1.3 supported the notion that large body size is a factor influencing mate choice, large males paired with large females (N=67) often than small females (N=12), which either large or small females has equal chances to mate with males.

In experiment 2, I demonstrated that large males probably prefer to mate with large females first when they have a chance to choose their partner. However, when the holding period was longer, no significant differences were found by female size, suggesting that polygynous males eventually mated with the smaller females (Roth 1948, Jones and Wheeler 1965, Spielman et al. 1967, Jones 1973). A recent study of male seminal depletion for *Ae. aegypti*, (Helinski and Harrington 2011) demonstrated that large and small males are capable of mating sequentially with up to 3-5 females, respectively over an 8 hour period, but female fecundity was reduced with partially depleted males later in the matings sequence.

I intentionally kept the density of males to females low in this study to reflect natural conditions. In nature, sex ratios of this species are closer to 1:1 and the average numbers of males found resting inside houses can range from 0 to 20 and varies considerably by house and region of the world (Harrington et al. unpublished data from Thailand, Mexico, and Puerto Rico, Scott et al. 2000, Garcia-Rejon et al. 2008). Although I used naturally low densities, this led to smaller than optimal samples limiting statistical power. Using a post hoc sample size estimate calculation with a 95% level of confidence and estimated proportion of large (0.625) and small (0.375) females chosen by males, I would need at least 40 females for the 60 min time period and 60 females for the 90 min time period in order to have sufficient statistical power. However this higher density of mosquitoes (~ 8 to 12 mosquitoes/m^3) is rarely found in natural conditions and may have affected mosquito behavior. Despite this dilemma, future studies should be conducted in larger cages to expand the mosquito density per m^3 such a whole empty room in house.

My final experiments involved a combination of semi-field free-ranging enclosures and stable isotope semen labeling to examine assortative of mating patterns in *Ae. aegypti*. The stable isotope method has been used to label male semen in other mosquito studies and can be reliably detected in female spermathecae using mass spectrometry (Helinski et al. 2007, 2008). More recently, Helinski et al. (2012) confirmed the value of this approach for *Ae. aegypti*. In my study, both types of labeled males obtained similar numbers of female mates, suggesting that there was no effect of either label on mating performance. In this study, when large males were labeled with ^{13}C, they mated with large females more often than small ones. However, when the isotope label was switched to large males labeled with ^{15}N, no significant of the mating pattern was observed. As a consequence, without further replication, I cannot determine if body size assortative mating occurs in *Ae. aegypti* under these conditions. Genetic markers could provide more efficient and less costly approaches to identifying paternity; however genetic markers have yet to be successfully applied to paternity studies in *Ae. aegypti* they are often described but not tested in the field or used for other purposes (Slotman et al. 2007, Chambers et al. 2007, Lovin et al. 2009, and Brown et al. 2011). When I conducted a post hoc estimate of sample size for proportions based on a 95% level of confidence and an estimated proportion of 0.53 (male matings with large females) and an error margin of ± 0.2, I determined that I would need 24 females per replicate instead of 10 used in my study in order to detect a real difference. With smaller margins of error such as ± 0.1, I would need 96 females. Future studies could also be conducted with more body sizes of both sex categories (such large, medium, and small size) to represent more of the range of sizes in natural population.

In conclusion, I have shown some indication that mate choice by size may occur in *Ae. aegypti*. However, more experiments need to definitively address this question.

FUTURE DIRECTIONS

AGs-specific promoter

The AG-specific promoter may be a useful tool for predicting function of seminal fluid proteins if it can be used to successfully drive desired expression in *Ae. aegypti*. This promoter may be used to study gene function of AGs genes by over-expressing or knocking down genes of interest.

Factors influencing mating success in *Ae. aegypti*

As many factors may influence mating success in this species, future research should focus on determining factors lead to mating success in dengue endemic region. My study provided some evidence of male sperm allocation patterns to females with different body size; however, the mechanism of allocation warrants further investigation. More studies should be conducted on mating preference or mate choice in this species. Collectively, understanding aspects of basic vector biology such as mechanisms of male preference may benefit use of genetically modified mosquito control strategies for vector control.

APPENDIX

Table A1. Sequencing results of the predicted promoter region.

Differences in: contig1	seq. ID								Total
Base pair Position		241	587	1796	2983	3722	3725	4778	differences
Consensus		G	T	G	T	A	G	T	
5 Kb upstream 10824 vectorbase		G	T	G	T	A	G	T	0
10824/male2_TOPOXL_no.8	1	A	G						2
10824/male1_TOPOXL_no.5	2	A	G						2
10824/male2_TOPOXL_no.8	3	A	G						2
10824/male1_TOPOXL_no.5	4	A	G						2
10824/male1_TOPOXL_no.5	5	A	G						2
10824/male2_TOPOXL_no.8	6	A	G						2
10824/male2_TOPOXL_no.8	7			C					1
10824/male1_TOPOXL_no.5	8			C					1
10824/male1_TOPOXL_no.5	9			C					1
10824/male2_TOPOXL_no.8	10			C					1
10824/male1_TOPOXL_no.5	11			C					1
10824/male2_TOPOXL_no.8	12			C					1
10824/male2_TOPOXL_no.8	13								0
10824/male1_TOPOXL_no.5	14								0
10824/male1_TOPOXL_no.5	15				C				1
10824/male2_TOPOXL_no.8	16				C				1
10824/male1_TOPOXL_no.5	17				C	G	A		3
10824/male2_TOPOXL_no.8	18				C	G	A		3
10824/male2_TOPOXL_no.8	19					G	A		2
10824/male1_TOPOXL_no.5	20					G	A		2
10824/male1_TOPOXL_no.5	21							C	1
10824/male2_TOPOXL_no.8	22							C	1
Summary:		1G,6A	1T,6G	1G,6C	1T,4C	1A,4G	1G,4A	1T,2C	32

SPSS ANALYSIS RESULTS OF CHAPTER 3

Table A2. Mean and standard deviation of sperm number by treatment in replicate 1 and 2.

Sperm count

Treatment*	Mean	N	Std. Deviation
LN	405.1	30	274.3
SN	152.7	30	90.6
LC	291.7	30	137.6
SC	144.3	30	91.1
Total	248.4	120	86.8

* = large (LN), small (SN) females in the absence of male competition and large (LC), small (SC) females in the present of male competition.

Table A3. Separation of means of sperm number in replicate 1 and 2.

sperm count (Tukey B)

Treatment*	N	Subset for alpha = 0.05	
		1	2
SC	30	144.3	
SN	30	152.7	
LC	30		291.7
LN	30		405.1

Means for group in homogeneous subsets are displayed.
Uses Harmonic Mean Sample Size = 30.00.
* = large (LN), small (SN) females in the absence of male competition and large (LC), small (SC) females in the present of male competition.

Table A4. Mean and standard deviation of sperm number by treatment in replicate 3.

Treatment*	Mean	N	Std. Deviation
LN	2,763.4	15	801.1
SN	2,002.3	15	553.8
LC	2,643.9	15	706.3
SC	1,907.6	15	399.1
ILC	2,783.3	15	664.3
Total	2,420.1	75	624.9

* = large (LN), small (SN) females in the absence of male competition and large (LC), small (SC) females in the present of male competition including large females (ILC) in the presence of male (separated male after eclosion) competition.

Table A5. Separation of means of sperm number in replicate 3.

Sperm count Tukey HSD

Treatment*	N	Subset for alpha = 0.05	
		1	2
SC	15	1,907.6	
SN	15	2,002.3	
LC	15		2,643.9
LN	15		2,763.4
ILC	15		2,783.3
sig.		0.998	0.976

Means for group in homogeneous subsets are displayed.
Uses Harmonic Mean Sample Size = 30.00.
* = large (LN), small (SN) females in the absence of male competition and large (LC), small (SC) females in the present of male competition including large females (ILC) in the presence of male (separated male after eclosion) competition.

Table A6. Test of between subjects effects of AALE010824 intensity in replicate 1 and 2.

Dependent Variable: log transformed

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected Model	0.10[a]	23	0.004	0.715	0.819
Intercept	5.01	1	5.012	867.215	0.000
Gel	0.04	5	0.008	1.360	0.247
Treatment_ID	0.03	3	0.008	1.431	0.239
Gel*Treatment_ID	0.03	15	0.002	0.354	0.987
Error	0.52	90	0.006		
Total	6.36	114			
Corrected Total	0.62	113			

a. R squared = 0.154 (Adjusted R squared = -0.062)

Table A7. Test of between subjects effects of AALE010824 intensity in replicate 3.

Dependent Variable: Intensity with AG normolized

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	1.56[a]	15	0.104	1.876	0.066
Intercept	27.65	1	27.652	497.589	0.000
Treatment ID	0.35	3	0.116	2.081	0.122
Replicate	0.00	0	-	-	-
Gel number	0.38	3	0.127	2.281	0.098
Treatment ID * Replicate	0.00	0	-	-	-
Treatment ID * gel number	0.84	9	0.093	1.673	0.137
Replicate*gel number	0.00	0	-	-	-
Error	1.78	32	0.056	-	-
Total	30.10	48	-	-	-
Corrected Total	3.34	47	-	-	-

a. R squared = 0.468 (Adjusted R squared = 0.219)

Table A8. Test of between subjects effects of AALE010824 intensity in replicate 3 for males isolated after eclosion (ILC) and grouped males.

Dependent Variable: Intensity with AG normolized

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	9.98[a]	11	0.907	16.998	0.000
Intercept	45.30	1	45.297	848.856	0.000
Treatment	0.18	2	0.089	1.667	0.203
Gel	9.04	3	3.014	56.487	0.000
Gel number	0.38	3	0.127	2.281	0.098
Treatment * Gel	0.76	6	0.126	2.364	0.050
Error	1.92	36	0.053		
Total	57.20	48			
Corrected Total	11.9	47			

a. R squared = 0.468 (Adjusted R squared = 0.219)

Table A9. Separation for AALE010824 intensity for males isolated after eclosion (ILC) and grouped males (replicate 3).

Tukey HSD[a]

Gel number	N	Subset[b] 1	2	3
4	12	0.39		
1	12		0.73	
2	12			1.28
3	12			1.48
sig.		1.000	1.000	0.159

Means for group in homogeneous subsets are displayed.
Based on observed means. The error term is mean square(error) = 0.053
a. uses harmonic mean sample size = 12.00
b. Alpha = 0.05.

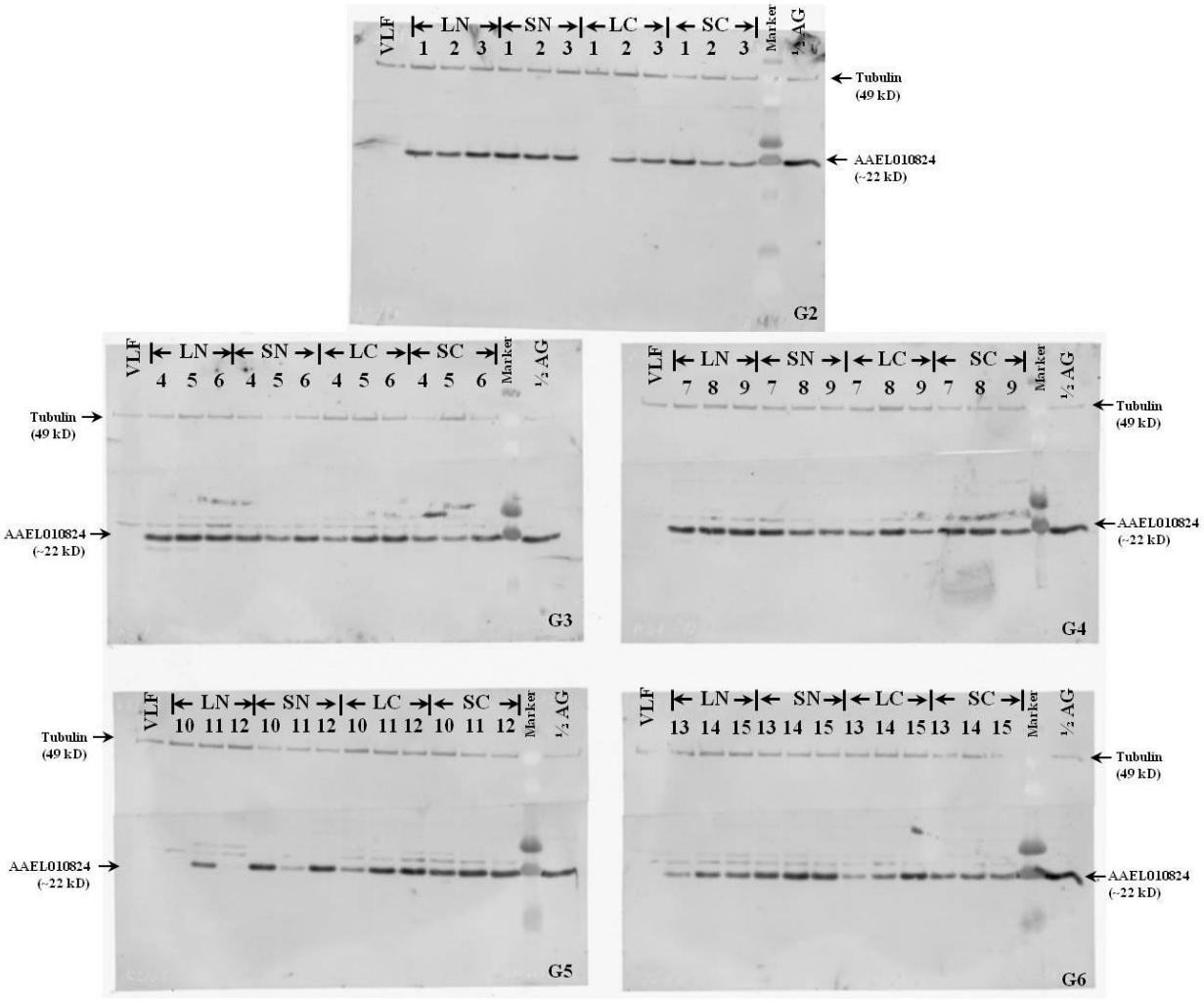


Figure A1. Western blot results of replicate 2. Treatment: large (LN) and small (SN) females in the absence of male competition, and large (LC) and small (SC) females in the presence of male competition (G2-G6 = gel 2-6); tubulin (49 kD) and AAEL010824 (~22kD), Precision Plus Protein™ Dual Xtra Standards marker (Bio-Rad). Lane 1-15 = lower abdominal segments of one female from each treatment, VLF = lower abdominal segments of a virgin large female from the control treatment, and ½ AG = A half accessory gland of virgin males.

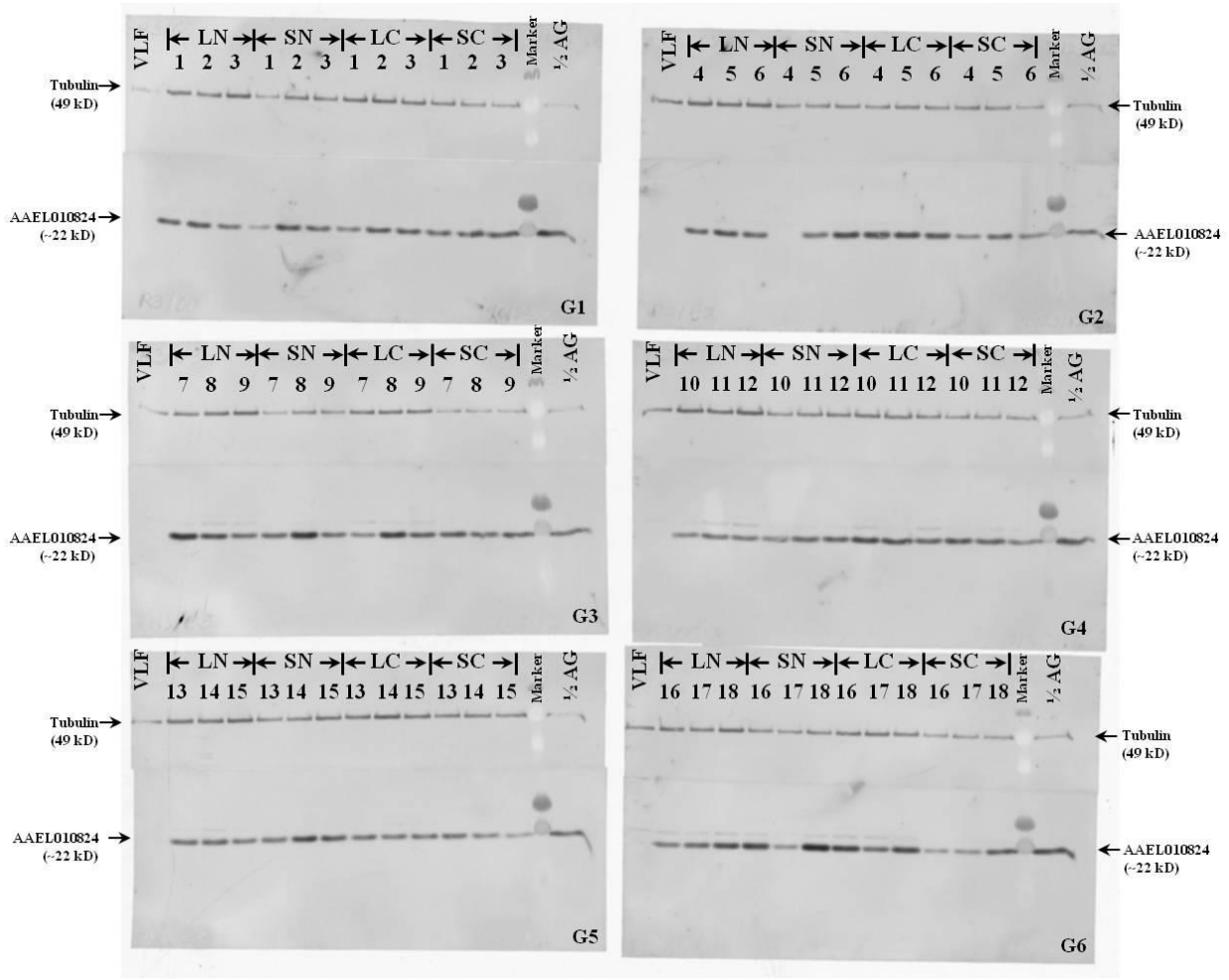


Figure A2. Western blot results of replicate 3A. Treatment: large (LN), small (SN) females in the absence of male competition, and large (LC) and small (SC) females in the presence of male competition (G1-G6 = gel 1-6); tubulin (49 kD) and AAEL010824 (~22kD), Precision Plus Protein™ Dual Xtra Standards marker (Bio-Rad). Lane 1-18 = lower abdominal segments of one female from each treatment, VLF = lower abdominal segments of a virgin large female from the control treatment, and ½ AG = A half accessory gland of virgin males.

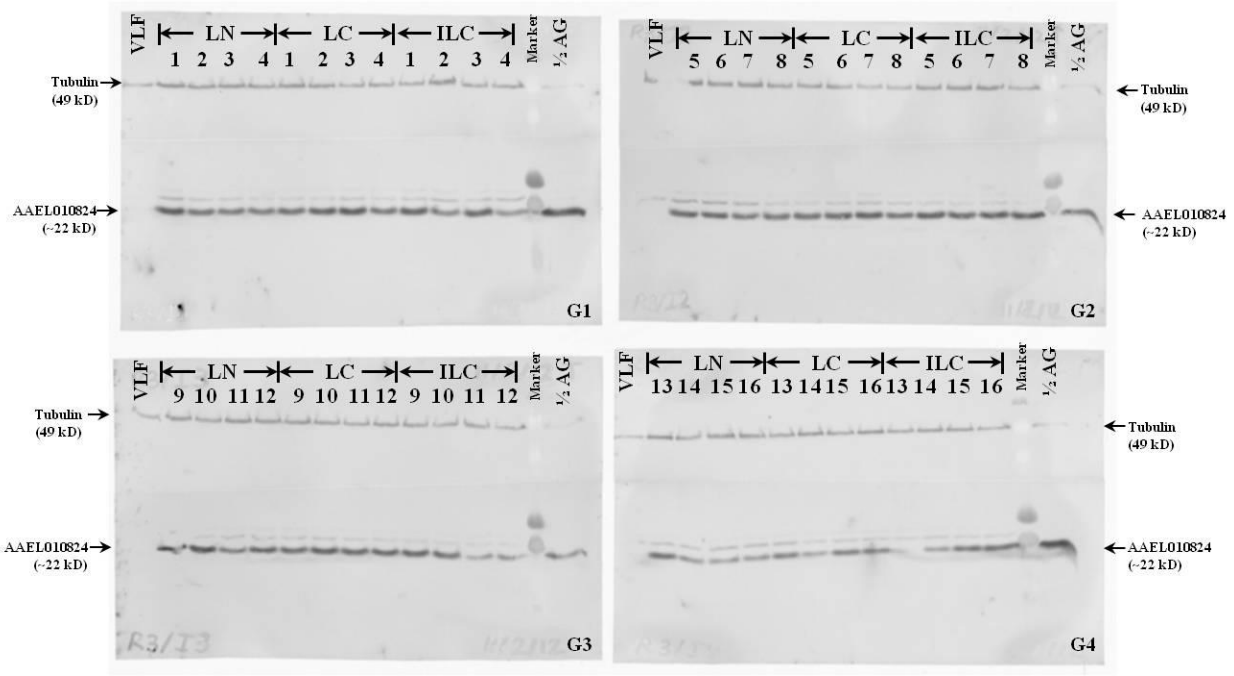


Figure A3. Western blot results of replicate 3B. Treatment (group housed males): large (LN) and small (SN) females in the absence of male competition, and large (LC) and small (SC) females in the presence of male competition. Treatment (male were isolated after eclosion until the experiment commenced): (ILC) large females in the present of male competition (G1-4 = gel 1-4); tubulin (49 kD) and AEL010824 (~22kD), Precision Plus Protein™ Dual Xtra Standards marker (Bio-Rad). Lane 1-16 =lower abdominal segments of one female from each treatment, VLF = lower abdominal segments of a virgin large female from the control treatment, and ½ AG = A half accessory gland of virgin males.

ISOTOPE ANALYSIS RESULTS OF CHAPTER 4

Table A10. Assortative mating using stable isotopes (^{15}N-labeled large males (^{15}N-LM) and ^{13}C-labeled small males (^{13}C-SM)).

Replicate	Sample ID of spermathecae	δ ^{13}C	C-amount (µg)	δ ^{15}N	N-amount (µg)
1	Blank	-25.88	40.70	1.34	30.73
	Mated large females no.1	-14.05	41.51	1.34	31.35
	Mated large females no.2	-25.91	40.70	6.81	30.88
	Mated large females no.3	-26.15	40.70	5.92	31.47
	Mated large females no.4	-14.44	40.70	1.12	31.31
	Mated large females no.5	-14.91	40.70	1.08	31.59
	Blank	-26.39	39.21	0.86	31.08
	Mated small females no.1	-20.45	41.10	1.10	31.63
	Mated small females no.2	-18.81	40.70	1.12	31.75
	Mated small females no.3	-26.14	40.70	4.52	32.10
	Mated small females no.4	-20.84	40.18	1.25	32.41
	Mated small females no.5	-16.45	40.70	0.97	32.77

Table A11. Assortative mating using stable isotopes (^{15}N-labeled large males; ^{15}N-LM and ^{13}C-labeled small males; ^{13}C-SM).

Replicate	Sample ID of spermathecae	δ ^{13}C	C-amount (µg)	δ ^{15}N	N-amount (µg)
2	Blank	-26.09	40.30	0.89	32.26
	Mated large females no.1	-25.96	37.07	10.06	29.35
	Mated large females no.2	-25.90	45.14	6.03	35.20
	Mated large females no.3	-15.22	40.70	1.05	32.18
	Mated large females no.4	-13.55	38.16	0.82	29.98
	Mated large females no.5	-26.26	38.44	6.51	30.25
	Blank	-26.29	37.19	0.99	29.31
	Mated small females no.1	-26.15	43.12	5.39	34.69
	Mated small females no.2	-11.96	39.93	0.88	31.67
	Mated small females no.3	-18.41	40.26	1.19	31.63
	Mated small females no.4	-25.93	40.13	6.65	31.67
	Mated small females no.5	-25.87	37.88	4.33	29.47

Table A12. Assortative mating using stable isotopes (^{15}N-labeled large males; ^{15}N-LM and ^{13}C-labeled small males; ^{13}C-SM).

Replicate	Sample ID of spermathecae	δ ^{13}C	C-amount (µg)	δ ^{15}N	N-amount (µg)
3	Blank	-25.60	35.94	0.80	26.65
	Mated large females no.1	-25.95	41.29	5.97	30.43
	Mated large females no.2	-22.23	41.29	4.89	30.28
	Mated large females no.3	-12.68	41.29	0.20	29.96
	Mated large females no.4	-25.76	41.29	6.00	30.28
	Mated large females no.5	-25.68	41.29	6.28	29.92
	Blank	-26.13	41.69	0.65	31.07
	Mated small females no.1	-26.06	40.88	3.81	30.00
	Mated small females no.2	-19.50	41.29	1.38	30.08
	Mated small females no.3	-17.71	41.29	1.15	30.63
	Mated small females no.4	-25.99	41.29	5.89	30.00
	Mated small females no.5	-25.79	42.50	4.27	29.68

Table A13. Assortative mating using stable isotopes (^{15}N-labeled small males; ^{15}N-SM and ^{13}C-labeled large males; ^{13}C-LM).

Replicate	Sample ID of spermathecae	δ ^{13}C	C-amount (µg)	δ ^{15}N	N-amount (µg)
1	Blank	-25.95	38.32	0.39	29.71
	Mated large females no.1	-26.06	40.70	5.60	31.20
	Mated large females no.2	-12.66	40.30	0.57	30.84
	Mated large females no.3	-17.12	40.70	0.81	31.24
	Mated large females no.4	-15.11	41.10	0.49	31.16
	Mated large females no.5	-15.83	40.70	1.56	30.06
	Blank	-25.86	36.54	0.35	28.37
	Mated small females no.1	-25.91	40.70	3.29	31.24
	Mated small females no.2	-26.07	40.70	2.63	31.16
	Mated small females no.3	-16.52	40.30	0.71	31.16
	Mated small females no.4	-25.95	40.70	5.37	31.27
	Mated small females no.5	-16.98	41.10	0.64	31.20

Table A14. Assortative mating using stable isotopes (^{15}N-labeled small males; ^{15}N-SM and ^{13}C-labeled large males; ^{13}C-LM).

Replicate	Sample ID of spermathecae	δ ^{13}C	C-amount (µg)	δ ^{15}N	N-amount (µg)
2	Blank	-25.94	38.76	0.38	29.86
	Mated large females no.1	-25.85	41.10	7.81	31.16
	Mated large females no.2	-14.27	40.70	0.50	31.04
	Mated large females no.3	-11.34	41.51	0.29	30.96
	Mated large females no.4	-17.48	41.10	0.73	31.24
	Mated large females no.5	-14.38	41.51	0.56	30.92
	Blank	-26.19	43.93	0.36	34.06
	Mated small females no.1	-23.59	40.70	4.36	30.84
	Mated small females no.2	-26.06	41.10	4.72	30.92
	Mated small females no.3	-26.23	41.10	6.95	31.00
	Mated small females no.4	-24.59	41.10	4.66	31.31
	Mated small females no.5	-19.15	41.10	0.80	31.12

Table A15. Assortative mating using stable isotopes (^{15}N-labeled small males; ^{15}N-SM and ^{13}C-labeled large males; ^{13}C-LM).

Replicate	Sample ID of spermathecae	δ ^{13}C	C-amount (µg)	δ ^{15}N	N-amount (µg)
3	Blank	-25.86	44.53	1.29	33.82
	Mated large females no.1	-	-	-	-
	Mated large females no.2	-18.86	41.29	1.30	30.24
	Mated large females no.3	-16.42	41.29	0.41	30.35
	Mated large females no.4	-15.10	40.48	0.89	29.52
	Mated large females no.5	-16.65	41.69	1.03	30.04
	Blank	-25.71	40.15	0.86	30.08
	Mated small females no.1	-25.89	41.69	7.69	30.24
	Mated small females no.2	-26.01	41.29	3.59	29.84
	Mated small females no.3	-25.86	38.17	6.20	27.61
	Mated small females no.4	-17.87	41.29	1.22	30.39
	Mated small females no.5	-21.47	42.10	0.52	30.04

Table A16. Controls for assortative mating using stable isotopes ^{15}N-labeled large males.

Control (females mated with ^{15}N-labeled large males) Sample ID of spermathecae	$\delta\ ^{13}C$	C-amount (µg)	$\delta\ ^{15}N$	N-amount (µg)
Large female no.1	-25.99	40.30	6.22	30.84
Large female no.2	-25.95	40.70	6.11	30.69
Large female no.3	-25.75	40.70	6.90	31.00
Large female no.4	-26.79	43.93	0.43	31.39
Large female no.5	-26.08	40.70	6.58	31.00
Large female no.6	-25.78	41.69	6.17	29.28
Large female no.7	-25.79	41.29	7.25	30.16
Large female no.8	-26.02	41.69	6.27	29.76
Small female no.1	-26.08	40.30	6.63	30.65
Small female no.2	-26.14	40.30	4.15	30.45
Small female no.3	-25.94	40.70	5.98	30.33
Small female no.4	-26.00	40.70	5.39	30.45
Small female no.5	-26.09	40.09	4.90	29.82
Small female no.6	-25.99	42.10	4.45	30.71

Table A17. Controls for assortative mating using stable isotopes ^{15}N-labeled small males.

Control (females mated with ^{15}N-labeled small males) Sample ID of spermathecae	$\delta\ ^{13}C$	C-amount (µg)	$\delta\ ^{15}N$	N-amount (µg)
Large female no.1	-25.80	41.29	6.15	29.64
Large female no.2	-25.69	40.88	9.05	30.23
Large female no.3	-25.74	42.10	10.90	29.60
Large female no.4	-25.79	40.88	6.74	30.12
Large female no.5	-25.89	40.88	4.80	30.23
Large female no.6	-26.06	42.10	7.79	29.60
Large female no.7	-25.74	41.29	5.56	29.32
Large female no.8	-26.12	41.69	8.14	30.19
Small female no.1	-26.12	41.29	2.84	30.63
Small female no.2	-25.98	40.88	5.59	30.27
Small female no.3	-26.01	41.29	9.96	30.43
Small female no.4	-25.93	40.88	0.59	30.31
Small female no.5	-25.88	42.91	3.72	30.47
Small female no.6	-25.77	41.29	6.50	30.08

Table A18. Controls for assortative mating using stable isotopes ^{13}C-labeled large males.

Control (females mated with ^{13}C-labeled large males) Sample ID of spermathecae	$\delta\ ^{13}C$	C-amount (µg)	$\delta\ ^{15}N$	N-amount (µg)
Large female no.1	-13.13	40.48	0.89	30.27
Large female no.2	-16.14	40.88	0.92	30.12
Large female no.3	-18.28	40.88	1.02	30.31
Large female no.4	-15.92	40.88	0.87	30.23
Large female no.5	-8.05	41.29	1.03	30.47
Large female no.6	-17.23	41.29	1.18	30.16
Large female no.7	-17.22	41.69	0.51	29.40
Large female no.8	-19.41	41.69	1.31	29.32
Small female no.1	-18.10	40.88	0.85	30.71
Small female no.2	-16.93	40.88	0.67	30.39
Small female no.3	-19.59	41.69	0.59	30.55
Small female no.4	-18.27	40.88	1.21	30.59
Small female no.5	-21.11	41.29	1.11	30.27
Small female no.6	-18.09	40.88	1.07	29.80
Small female no.7	-11.22	38.65	0.85	27.41
Small female no.8	-20.56	41.69	1.18	29.76

Table A19. Controls for assortative mating using stable isotopes ^{13}C-labeled small males.

Control (females mated with ^{13}C-labeled small males) Sample ID of spermathecae	$\delta\ ^{13}C$	C-amount (µg)	$\delta\ ^{15}N$	N-amount (µg)
Large female no.1	-17.60	40.30	0.45	30.41
Large female no.2	-13.42	40.30	0.88	30.53
Large female no.3	-14.04	40.30	0.66	30.25
Large female no.4	-14.59	40.30	0.34	30.02
Large female no.5	-16.97	40.30	0.69	30.37
Small female no.1	-19.68	43.52	0.70	30.45
Small female no.2	-21.14	40.70	1.06	30.10
Small female no.3	-19.29	40.30	0.23	29.94
Small female no.4	-18.30	40.01	0.58	30.22
Small female no.5	-11.99	40.21	0.43	30.25

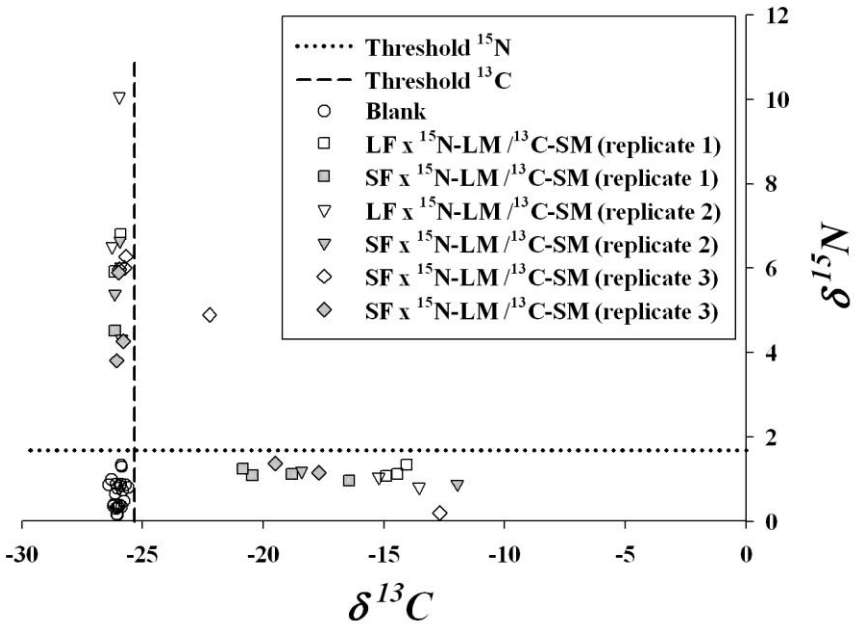


Figure A4. Three replicates of isotope analysis results (large and small females mated with ^{15}N-labeled large males or ^{13}C-labeled small males).

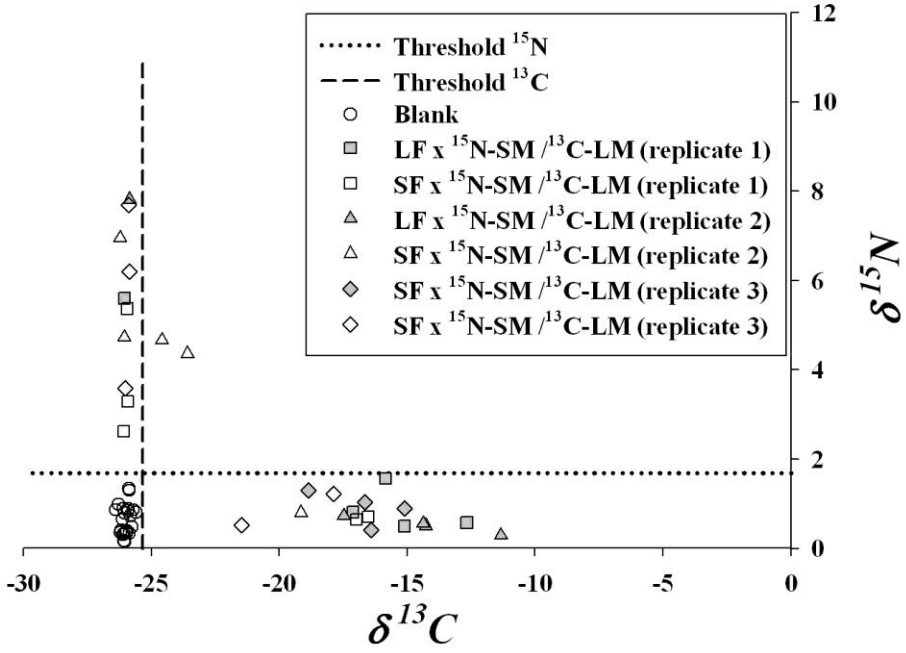


Figure A5. Three replicates of isotope analysis results (large and small females mated with ^{15}N-labeled small males or ^{13}C-labeled large males).

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